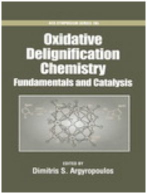

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Oxidative Delignification Chemistry

Fundamentals and Catalysis

EDITED BY

Dimitris S. Argyropoulos

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Oxidative Delignification Chemistry

Fundamentals and Catalysis

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Foreword

THE ACS SYMPOSIUM SERIES was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing, to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded in order to better focus the book: others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

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Preface

Despite the fact that the direct interaction of dioxygen with organic compounds is a highly exothermic and thermodynamically favorable process, such reactions are known to be rather slow, preventing all organisms from rapid combustion. Industrially it will be extremely beneficial to fix molecular oxygen within organic or inorganic compounds, mimicking nature's capability of transferring it selectively to an organic substrate such as lignin. It is in this context that the foundations for this book exist. The manufacturing process of white paper and related products requires a series of oxidative stages of considerable complexity and possible environmental load. Environmental regulations coupled to market pressures have forced the pulp and paper industry to explore alternatives to chlorine-based pulp-bleaching practices. Various technologies and chemicals have been suggested as answers to pulp bleaching concerns, from which oxygen, ozone, and peroxides are the leading candidates for chlorine and chlorine dioxide replacement. Unfortunately, none of these chemicals is without drawbacks. Exploratory attempts to improve the delignification and bleaching efficiency of oxygen and/or hydrogen peroxide commenced almost as soon as their delignification and brightening potential was realized. During the early efforts, the imposing limitations were economic and safety issues. During the past decade, however, environmental concerns and regulatory forces have seriously altered the way we view such improvements. Despite the renewed interest for developments in this area and our new outlook, many processes, activators, and catalysts fail to see widespread industrial applicability because the same economic and safety considerations still apply. The proper understanding of the fundamental organic chemistry of these oxidants with the lignocellulosic substrate could lead to process improvements with serious production and environmental consequences.

The literature is abundant with research accounts aimed at offering an understanding to these complex chemical processes. To date no concerted effort has been made about bringing this knowledge together with the aim to connect the past with the future. Consequently, at the turn of the twenty-first century (March 2000) an American Chemical Society (ACS) Symposium was held in San Francisco, California, focused at bringing together the global expertise from academia, government, and industry with the aim to disseminate their latest findings and exchange their ideas for the future. The present book attempts to offer the reader a thorough view of the information presented at this meeting. Despite the fact that the material emerged from a symposium,

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it is not a collection of fragmented research findings in the form of conference proceedings. Most chapter contributors have attempted to provide a good review of the literature, creating a sound foundation for the science to be subsequently developed. Furthermore, a collection of authoritative reviews is also provided prior to embarking on specific topics.

More specifically, the opening chapter of this book extensively reviews the research efforts aimed at utilizing oxygen and peroxide for removing the lignin from chemical pulps. Because these two traditional oxidants represent a major effort in our community, a relatively large fraction of ensuing chapters has been dedicated toward reviewing and exploring their organic chemistry and their fundamental interactions with the lignocellulosic substrate. Furthermore, the potential of catalytically activating hydrogen peroxide with biomimetic systems such as porphyrins and various transition metal complexes is explored. A number of promising research endeavors are documented using novel compounds and the emerging principles of organic and bioinorganic chemistry. Perhaps some of the answers may lie in the way nature reduces oxygen to water, controlling the process using various enzymatic systems and receptors. Understanding the principles of biomimetic oxidations and applying them could conceivably offer some truly revolutionary processes that may guide our industry in the new millennium. For this reason, no book of this nature would have been complete if the chemistry and potential applications of various oxidative enzymes were not addressed.

In an effort to convey the material in a coherent fashion the book has been divided into the following distinct sections:

- Oxygen and Peroxide Delignification
- Biomimetic Systems and Polyoxometalates
- Enzymatic Delignification Systems

The editor anticipates that this volume will provide a resource for new ideas, guidance, and a good embarkation point for any future endeavors in "*Oxidative Delignification Chemistry.*"

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This compilation is a product of a concerted effort of numerous individuals to whom the editor expresses his appreciation. Primarily, thanks are due to the many scientists who have contributed their time and effort in documenting their research findings. Without their enthusiastic response and support this volume would not have been possible. In addition, the monetary and other functional contributions of the Cellulose, Paper, and Textiles Division of the ACS toward organizing the aforementioned symposium is gratefully

acknowledged. No book of this nature would have been possible if it were not for the support of nearly 100 members of the international scientific community who acted as critical reviewers to the submitted chapters. Their invaluable critical and constructive comments ensured that each chapter was of the highest scientific standard, reflecting the state of the art, and was presented in the best possible way. Last but not least, the numerous secretarial contributions of Liz Ansell are gratefully acknowledged.

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Chapter 1

Catalysis and Activation of Oxygen and Peroxide Delignification of Chemical Pulps: A Review

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Oxygen and hydrogen peroxide have always been technologically attractive oxidants to the pulp and paper industry. The fact that molecular oxygen has a triplet ground state whose direct interaction with singlet-state organic molecules is a spin-forbidden transition, limits its oxidative selectivity. Industrially, it would be extremely beneficial to fix molecular oxygen within organic or inorganic compounds capable of transferring it selectively to an organic substrate such as lignin. Since a variety of research endeavours have already been made to catalyse oxygen delignification and activate peroxide delignification of chemical pulps, this paper critically reviews them. In addition, this effort covers peracids, which can be considered as organic molecules containing active oxygen. Dioxiranes have also been shown to possess the ability to transfer a single activated oxygen atom onto aromatic and unsaturated substrates. As such, dimethyldioxirane is reviewed for its potential as a novel and selective bleaching agent for the production of fully-bleached totally chlorine-free (TCF) pulp. Finally, our critical review covers the recent scientific and patent literature which contains a number of examples where transition metals have been used as additives in peroxide and oxygen delignification.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Introduction

Environmental regulations coupled to market pressures have forced the pulp and paper industry to explore alternatives to chlorine based bleaching practices for the production of bleached kraft pulp. Various technologies and bleaching chemicals have been suggested as answers to kraft pulp bleaching concerns, from which oxygen, ozone and peroxides are the leading candidates for chlorine replacement. Unfortunately, these chemicals are not without drawbacks.

Oxygen delignification is effective but its delignification effectiveness is limited to about 50% after which a severe loss of pulp strength occurs. Hydrogen peroxide under alkaline or acidic conditions is not an effective delignifying agent while ozone is considerably more effective but it promotes aggressive radical reactions which cause undesirable carbohydrate degradation.

High brightness pulps appear to be difficult to produce only by applying successive oxygen and peroxide stages. Repeating the same chemistry, stage after stage, gives diminishing returns, with the necessity to introduce stages that activate the pulp or catalyze the chemical used, before additional oxygen or peroxide becomes effective. Devising methods for such activation or catalysis has been the subject of many research endeavors whose specifics are reviewed in this paper.

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Activation of Oxygen Delignification

Oxygen delignification can be considered as an intermediate step between kraft pulping and bleaching since up to about 50% of the residual lignin in kraft pulp can be removed at this stage. Extending a conventional oxygen delignification stage beyond its current limits would decrease bleach chemical demand with serious environmental benefits and significant increases on return on investment.

Recent efforts to augment the efficiency of oxygen delignification can be classified as follows:

-
- Reinforcement, using peroxide or peracids
- Addition of activators or catalysts
- Pulp pretreatment prior to oxygen delignification
- Two stage oxygen delignification with or without interstage treatment, where the oxygen and alkali charges are split in two stages.

Peroxide and Peracids Reinforced Oxygen Delignification

Combining the effects of hydrogen peroxide and oxygen for the bleaching of chemical pulps was first reported in the 1980's. More specifically, a synergy between oxygen and peroxide was reported when peroxide was used for the reinforcement of alkaline extraction stages carried out under moderate oxygen pressures (Eop) (*1*, *2*). These data showed reduced kappa numbers and improved viscosities. Consequently, in an effort to improve the oxygen delignification stage itself, peroxide reinforced oxygen delignification was proposed aimed at enhancing the efficiency of lignin removal. Parthasarathy and co-workers showed that peroxide had an additive effect with oxygen on the delignification of pine kraft pulps, resulting in improved selectivity and delignification efficiency (*3*). For example, the addition of 0.5% hydrogen peroxide into an oxygen delignification stage, caused a 60% kappa number reduction as compared to a 50% maximum kappa number reduction possible for one stage oxygen delignification. Compared to conventional oxygen delignification, peroxide reinforcement (at lower alkali charges) produced delignified pulps of the same kappa number with higher viscosity than those obtained in its absence. In addition, peroxide reinforced oxygen delignification was found to allow the use of lower temperatures with similar kappa number reductions.

Parthasarathy and co-workers also examined the peroxide reinforcement of a double stage oxygen delignification process (*3*). Using a (PO)(PO) process, kraft pulps were shown to be delignified to about 73% of their original kappa number as compared to 61% for a double oxygen delignification process, in the absence of peroxide. Once again, at similar kappa numbers, peroxide reinforced double oxygen delignification produced pulps with higher viscosities and better strength properties. It was also postulated that peroxide has to be added during the first stage of a double stage oxygen bleaching to realize potential benefits from the second oxygen stage which may or may not be reinforced with hydrogen peroxide.

In another effort Odermatt and co-workers investigated the effect of adding hydrogen peroxide and activated hydrogen peroxide on the selectivity and delignification efficiency of oxygen delignification (*4*). By controlling the peroxide decomposition with diethylenetriamine pentaacetic acid (DTPA) and an acid pretreatment, oxygen delignification in the presence of cyanamide activated hydrogen peroxide, resulted in pulps of low lignin content (kappa numbers bellow 10, with over 60% delignification), while maintaining good selectivity. Applying two oxygen treatments reinforced with cyanamide activated hydrogen peroxide, i.e. an (OP)(OP) sequence, a pulp of kappa number 4.4 (amounting to an 82% overall delignification) was produced.

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Peroxide activated with polypyridines (*5*) as well as peracids, have been examined by different groups as potential systems for reinforcing oxygen delignification. These systems will be described in detail in other sections of this paper.

Oxygen delignification of kraft pulp in the presence of acidic peroxide was originally described by Süss and Helmling in 1987 (*6*). Such a treatment was shown to improve the delignification rate of a subsequent alkaline stage. Further efforts addressed the addition of molybdate ions in acidic peroxide assisted oxygen delignification (*7*). An addition of a 1% peroxide together with molybdate (300 ppm as Mo) was found to reduce the kappa number to 10.3, as compared to a kappa number of 16.8 of the control experiment. Serious viscosity losses, however, accompanied these effects.

Liebergott studied peracid/oxygen treatment of kraft pulp (*8*). Acidic peroxide (AP), peracetic acid (Pa), peroxymonosulfuric acid (Px) and mixed peracids solution (Pxa) were tested under acidic conditions with oxygen on an oxygen delignified softwood pulp (kappa number 20). Px/O and Pxa/O treatment followed by Eop extraction resulted in a delignification of 63% (kappa 7.5) and 67% (kappa 6.7) respectively.

Polyoxometalates and Other Transition Metal Based Activators

Extensive research on polyoxometalates (POM) bleaching commenced in 1992 by the USDA Forest Service, Forest Product Laboratory (*9, 10*) and in 1995 a highly selective closed mill technology was claimed by the group (*11, 12, 13*). The process cycle starts with the reaction of fully oxidized POM with unbleached kraft pulp under anaerobic conditions (eq. 1). At this stage the POM complexes are reduced, and oxidized residual lignin fragments are dissolved in the bleaching liquor. After the bleaching, the reduced POM liquor can be re-oxidized in a separate stage (eq. 2) using oxygen. During the regeneration of the POM complexes the dissolved lignin fragments are converted to carbon dioxide and water. The recycling potential of the POM liquor has been demonstrated (*11*).

$$\text{Pulp} + \text{POM}_{\text{ox}} \rightarrow \text{Bleached pulp} + \text{POM}_{\text{red}} \qquad (1)$$

$$\text{POM}_{\text{red}} + O_2 + H_4^+ \rightarrow \text{POM}_{\text{ox}} + 2H_2O \qquad (2)$$

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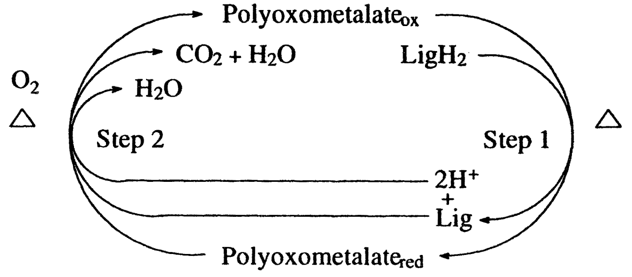
Recently, two different POM anions (α-$[SiVW_{11}O_{40}]^{5-}$ and α-$[PV_2Mo_{10}O_{40}]^{5-}$) were selected as potential catalysts of oxygen delignification and extensive studies of their reactions with phenolic lignin model compounds have been reported (*14*, *15*).



Scheme 1. An effluent free POM delignification process. Step 1: Anaerobic oxidation of lignin in wood or pulp fiber by fully oxidized POM complexes (POM$_{ox}$); Step 2: Aerobic wet oxidation (mineralization) of dissolved organic compounds and regeneration of the reduced POM complexes (POM$_{red}$) to their fully oxidized, delignification-active form. (14)

Another generation of POMs has recently been developed by Atalla and co-workers (*16*). While the first generation was able to operate only at acidic pH, the new POMs are stable at pH levels above neutrality. In this respect the hydrolysis of cellulose is significantly reduced. In addition the group claimed that this new generation of POM's is of improved stability due to newly developed synthetic procedures, which allow the POM to be repeatedly recycled in a closed system. More specifically, POM with the formulae $Na_6SiV_2W_{10}O_{40}$ was shown to be effective in reducing the kappa number of unbleached kraft pulp from above 30 to below 10 with limited losses in viscosity.

In contrast to the anaerobic reaction conditions required for the use of POM's developed by the Weinstock and Atalla team, Evtuguin and Pascoal Neto (*17*) have described the possibility of using POM catalysts under aerobic conditions. In this respect, the possibility of oxygen delignifying pulp in mixtures of organic solvents with water in a single-stage aerobic process has been patented (*17*). The oxidation of lignin under an oxygen atmosphere occurs *via* its reaction with POM while at the same stage the re-oxidation of a reduced form of POM by oxygen takes place. The presence of such a redox cycle could conceivably offer good process efficiencies. In actual fact, preliminary data of

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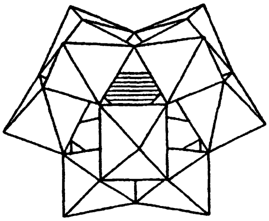
POM assisted oxygen pulping of eucalyptus wood and POM assisted oxygen bleaching of sulfate pulp in organic solvent/aqueous media have been presented (*18*). More specifically, the heptamolybdopentavanadophosphate (HPA-5) anion was shown to be promoting oxygen delignification in organic solvent/aqueous media.



Scheme 2: The α-Keggin structure of the 12-series heteropolyanion (HPA) in polyhedral-filling representation. (19)

A mechanistic proposal and possible applications of HPA-5 have been reported by Evtuguin and co-workers (*20*), and the catalytic effect of HPA-5 for the oxygen delignification of kraft pulp has been documented (*21*). More specifically, oxygen delignification experiments with eucalyptus kraft pulp in the presence of HPA-5 showed that the degree of delignification depends on the nature of the solvent used. Water, acetone/water, and ethanol/water were examined as delignification media and the properties of the bleached pulps were compared. While the degree of delignification was highest in aqueous media the selectivity of delignification was found to increase with the addition of organic solvent (ethanol). This effort defined the optimal delignification conditions as follows: Temperature: 90°C; Reaction time: 2 hrs, Solvent composition: 50/50 ethanol/water; pH: 1.8 and concentration of HPA-5: 2mmol/L. Notably, no significant changes in the efficiency of the catalyst were observed after recycling the same material up to ten times. The use of polyoxometalates has also been extended to elemental chlorine free (ECF) and totally chlorine free (TCF) bleaching sequences (*22*). Pulps of high brightness, acceptable strength properties and reduced requirements of ClO_2 have been claimed. POM systems were also reported to activate peroxide and ozone bleaching stages of eucalyptus kraft pulp under acidic conditions (*22*). While HPA-5 was found to catalyze peroxide delignification, the low pH's used resulted in significant pulp viscosity losses.

Perng and co-workers examined the effect of water soluble metal porphyrins toward improving the rate and selectivity of oxygen delignification

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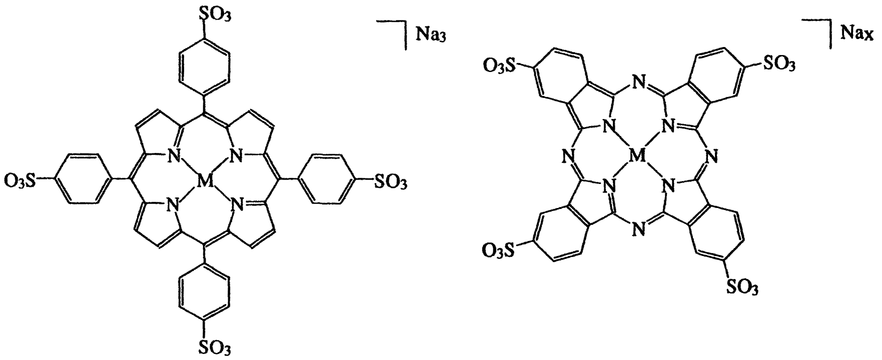
for softwood kraft pulp (*23*). Amongst the cobalt, iron and manganese complexes of meso(terasulfonatophenyl) porphyrin (TSPP) and tetrasulfopthalocyanine (TSPc), only Mn(TSPP) showed catalytic activity by promoting delignification and suppressing cellulose degradation (Scheme 3). The iron complexes appeared to suppress cellulose degradation, while the cobalt complexes were found to promote it with no effect on the delignification efficiency.



Na$_3$M(TSPP)
M = Co(III), Fe(III), Mn(III)

Na$_x$M(TSPc)
M = Co(II), x = 4, Fe(III), x = 3

Scheme 3: Water-soluble metal porphyrin and phthalocyanine complexes as potential oxygen delignification catalysts (23)

Transition metals and transition metal complexes have also been investigated as potential additives acting as catalysts or activators for oxygen delignification of kraft pulps (*24, 25*). Ni^{2+} ions in the presence of sodium tartrate were found to increase the delignification selectivity and similar effects were observed when the tartrate was substituted with equal amounts of sodium oxalate or sodium citrate. The enhanced selectivity was attributed mainly to reduced carbohydrate degradation since the extent of oxygen delignification was only marginally increased.

Recently, Agnemo showed that molybdate addition to an acidic (pH~5) softwood oxygen delignification stage resulted in increased kappa number reduction (*7*). More specifically, a pulp of kappa number of 11.2 was obtained when 300 ppm of molybdate ions were added during the delignification as opposed to a kappa 16.8 obtained for the control.

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Pulp Pretreatment Prior to Oxygen Delignification

A number of investigators have reported on the beneficial effects of kraft pulp pretreatment prior to an oxygen delignification stage. Pretreatments with compounds such as nitrogen dioxide (*26, 27, 28, 29*), chlorine (*30, 31, 32*) chlorine dioxide (*30, 31*) and hydrogen peroxide (*30*) were shown to improve oxygen delignification by increasing the selectivity of the delignification allowing for extending the delignification without significant losses in pulp quality.

Pretreatment of kraft pulp with nitrogen dioxide has been shown to promote oxygen delignification to a very low lignin content without causing extensive depolymerization of cellulose. Early efforts by Abrahamsson and co-workers have shown that nitrogen dioxide pretreatment of kraft pulp causes partial demethylation of the lignin (*26*). In addition, model compound work by Lindeberg and Walding has shown that NO_2 treatment caused the fragmentation of ether bonds within residual lignin with the consequent formation of new free phenolic groups (*33*). These reactions are expected to increase the reactivity of the lignin and decrease the possibility of cellulose being attacked by oxygen and radical species. In addition these reactions are thought to contribute to the alkali solubility of the lignin, in a sodium hydroxide impregnation stage, prior to oxygen delignification (*26*). At an applied front, work by Lindqvist and co-workers showed that the nitrogen dioxide pretreatment significantly assisted an oxygen delignification stage by reducing the kappa number while retaining pulp viscosity (*27*). Samuelson and Öjteg showed a dramatic increase in the efficiency and the selectivity of oxygen delignification when softwood kraft pulp was subjected to a two-stage NO_2 pretreatment process, followed by an oxygen delignification stage (kappa number 3.5) (*28*). It is worth mentioning at this point that, the nitrogen dioxide pretreatment described above has actually been evaluated and implemented on a pilot scale in Sweden (*34*).

Interesting carbohydrate protection effects have been noted when kraft pulp was pretreated with a low charge (<2%) of chlorine. Up to 75% of the residual kraft lignin was found to be removed in a subsequent oxygen stage, while the pulp properties were maintained. Several investigators have independently confirmed the beneficial effects of such a chlorine pretreatment allowing for extensive delignification (kappa number below 10) in a subsequent oxygen delignification stage (*35, 36, 30*). The hypothesis offered to rationalize for these data was that chlorine causes the generation of new phenolic groups, which would allow for a more selective reaction of oxygen with lignin. Chlorine dioxide was also shown to have similar effects at a given active chlorine charge (*30*). This is possibly a more attractive option from an environmental and a technological point of view, since chlorine dioxide produces only one fifth of

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the chlorides usually produced by an equivalent amount of chlorine. Chlorine dioxide is also considerably less corrosive than chlorine. However, both of these chlorine containing activation systems add significant environmental load to the process of oxygen delignification since chloride ions are introduced in the effluent, prohibiting its recovery.

The activating effect of ozone was also studied by Lachenal and de Choudens (*35*) as a pretreatment to oxygen delignification. While these authors reported small improvements, Fossum and Marklund found no synergistic effects operating on the selectivity between the ozone pretreatment and the oxygen stage (*30*).

Pulp pretreatment with acidic hydrogen peroxide has been documented to offer benefits as far as the selectivity of an oxygen delignification stage is concerned, although the effect was always lower than that of NO_2 or chlorine. These effects were seen to be significantly reduced when the peroxide was applied under neutral conditions (*30*).

Pretreatments with sulphur dioxide, sodium sulphite, and sodium hypochlorite have also been examined by Fossum and Marklund, but no significant effect on the selectivity of subsequent oxygen delignification stages was observed (*30*). Improved selectivity was however, observed by Andrews and co-workers after sulphide containing green liquor was used as a pretreatment of kraft pulp prior to oxygen delignification (37). Fossum and Marklund, however, showed that no selectivity improvements were apparent when sulphide was used as a pretreatment compared to conventional oxygen delignification (*30*).

The use of peroxyformic acid, generated *in-situ* from a mixture of formic acid and hydrogen peroxide, prior to oxygen delignification allowed a total degree of delignification of 83.4% to be attained without any serious drop in viscosity (*38*). Kappa numbers as low as 4-6 were obtained for both pine and birch kraft pulps after the (PFA)O stage. Prior to the process, however, metals had to be removed in order to prevent the decomposition of the peroxyformic acid. The reasoning behind this impressive degree of delignification was rationalized by Poppius *et al.* as being due to increased reactivity of the residual lignin toward oxygen delignification, allowing for a substantial decrease in the kappa number in the oxygen stage.

Springer and McSweeny found that peroxymonosulfuric acid pretreatment prior to oxygen delignification was equally effective to that of chlorine provided that the transition metals were removed prior to the treatment (*39*). Süss and Helmling invoked oxidation and hydroxylation reactions occuring between

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peroxymonosulfuric acid and the aromatic rings of lignin. These reactions are thought to increase the number of free phenolic groups in lignin thus increasing the number of sites available for oxygen attack during the alkaline oxygen delignification stage (*6*).

Double Oxygen Delignification with Interstage Treatment

A two stage oxygen delignification process was designed by Kleppe and Peterson aimed at improving the delignification effectiveness of medium consistency oxygen stages, by dividing the oxygen and the alkali charge in two separate stages using two separate mixing systems. It was thus possible to increase the rate of lignin removal while preserving the degree of polymerization of the cellulose (*40*). The delignification efficiency of the two-stage oxygen delignification was found to be further increased by maintaining a high pH, by adding fresh alkali and remixing the pulp before the second oxygen stage (*41*). A cumulative degree of delignification of about 70% was claimed when double stage oxygen was applied in the absence of interstage washing, compared to 53% delignification usually attained during a single oxygen stage.

A number of recent studies have shown that the structure of the residual lignin is significantly altered after oxygen delignification making the residual lignin less reactive to another oxygen stage (*42*, *43*, *44*). Efforts to increase the extent of delignification during the second oxygen stage, have been centred around modifications of the residual lignin aimed at increasing its reactivity toward oxygen. In addition, various pretreatments aimed to activate the lignin prior to oxygen delignification have been reported with marked improvements in selectivity. These pretreatments include nitrogen dioxide (*26*), ozone and peroxide (*30*), sulphur dioxide, sulphite and sulphide (*30*), hypochlorite (*30*), chlorine and chlorine dioxide (*31*), peracids (*45*), and even enzymes (*46*).

In an attempt to combine the benefits of lignin activation with the benefits of a two-stage oxygen delignification process, a two-stage oxygen delignification with an interstage chlorine (X) treatment, was proposed by Lachenal *et al.* in 1989 (*31*). The OXO process was more extensively studied and optimized two years later by Lachenal and Muguet (*32*). In actual fact, the presence of chlorine (1.5%, active chlorine) between the two oxygen stages provided a well delignified softwood kraft pulp with improved strength properties since the selectivity of the second oxygen stage was significantly improved. The efficiency of chlorine and chlorine dioxide as activating stages was also compared. Chlorine was found to be more efficient than chlorine dioxide in inducing the sought activation. Finally, a mill-scale implementation of the OxO process was reported in 1990 (*47*). Despite the advantages

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enumerated above the use of chlorine is accompanied by the formation of organochlorines and corrosive chlorides in the process effluent, making the process less attractive from an environmental point of view.

Allison and McGrouther attempted to further improve the two-stage oxygen delignification process using an interstage treatment with peroxymonosulfuric acid (Px) *(48)*. A charge of 2% of peroxymonosulfuric acid was found to improve the overall selectivity of an OPxO sequence. Using this combination of chemicals it became possible to delignify the pulp at a 70% level with better viscosity than a comparable pulp delignified at the same kappa number with a conventional OO sequence. The Px treatment was effective under both acidic (pH 4) and alkaline (pH 10) conditions at moderate temperatures (75°C) at contact times of about 30 min. By adding hydrogen peroxide in the product solution of an on-site generated peroxymonosulfuric acid it was found that it enhanced the overall selectivity of the process. The presence of hydrogen peroxide was actually found to be synergistic with peroxymonosulfuric acid. Interstage treatment with peroxide alone had no effect on selectivity, and treatment with peroxymonosulfuric acid alone (in the form of triple salt of potassium peroxymonosulfate) improved the selectivity but not to the same extent as the treatment with the combination of both oxidants. A more detailed study of the effect of hydrogen peroxide on OPxO was reported by Allison and co-workers (*49*). The authors showed that no benefits to the overall process selectivity were observed when hydrogen peroxide was present in the Px stage. The presence of peroxide in the final oxygen stage of the OPxO process, however, was found to be beneficial. This contradiction to the conclusion of the earlier work of Allison and McGrouther *(48)* has been caused by residual chemical carryover. Most of the original hydrogen peroxide in interstage treatment mixture survived the short Px interstage and was thus present during the final oxygen delignification stage. Subsequently, the presence of hydrogen peroxide in the second stage improved the overall delignification efficiency of the OPxO process, not the presence of hydrogen peroxide in the Px interstage itself.

Also studied was the effect of metal ions on the selectivity of the treatment. Metal ions were found to catalyze the reactions causing cellulose degradation when present in peroxymonosulfuric acid treatments. Direct addition of the chelating agent to the Px stage did not, however, minimize the adverse effects of the metal ions. As such it was concluded that metals must be removed (an ethylenediaminetetracetic acid - EDTA pretreatment followed by wash) prior to the Px treatment. The ideal operating conditions of the OPxO bleaching sequence were found to be metal removal prior to the peroxide free Px treatment, washing after the Px interstage, and peroxide reinforcement of the second oxygen delignification stage. Initial pilot scale trials confirmed that very

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low kappa numbers for hardwood and softwood kraft pulps could be obtained *via* an OQPx(OP) sequence. However, careful control of the peroxymonosulfuric acid reactions is required since extensive reaction during the Px stage could reduce the overall selectivity of the process.

In an another modification, McGrouther and Allison studied the addition of acetone to the peroxymonosulfuric interstage treatment with the aim to further increase the two-stage oxygen delignification selectivity (*50*). It was found that the addition of acetone was beneficial only at HSO_5 charges higher than 2.5%. Below these levels the peroxymonosulfate reaction with acetone, known to *in situ* form Dimethyldioxirane (DMD), appeared to be unable to compete effectively with the peroxymonosulfate reactions with the pulp. McGrouther and Allison also examined the effect that acetone addition has on the brightness development of the OPxO bleaching sequence, since earlier studies showed that peroxymonosulfate effectively delignified the pulp without, however, promoting subsequent brightening stages (*51*). An interstage treatment with Px(5%)/acetone(4%), followed by a second oxygen delignification stage with 3% NaOH, resulted in an increased final pulp brightness when compared to the control OPxO pulp. This impressive data was rationalized on the basis that *in situ* produced DMD was able to eliminate chromophores to a greater extent than peroxymonosulfuric acid. In addition to these studies Allison's team also examined the effect of enzyme pretreatment prior to the Px stage on the overall OPxO effectiveness and selectivity (*52*). More specifically, in this study, oxygen delignified kraft pulp was pretreated with combinations of a commercial xylanase enzyme and a chelating agent prior to the peroxymonosulfate treatment and the oxygen stage. The data showed that the use of enzyme increased the efficiency of the subsequent peroxymonosulfate delignification stage with little viscosity loss. The resulting increase in the overall OPxO selectivity allowed a decrease of up to 25% in the peroxymonosulfate charge required to obtain an OPxO pulp of a given kappa number and viscosity.

Hunt and Lee examined dimethydioxirane (T) as an interstage treatment preceding the second oxygen delignification stage in an OTO bleaching sequence and compared the efficiency of DMD with chlorine dioxide in the ODO bleaching sequence (*53*). DMD was found to significantly enhance the kappa number reduction in the second oxygen stage. It was also shown that DMD is of the same selectivity as chlorine dioxide. As an interstage treatment, DMD showed a greater brightness gain per kappa unit reduction than chlorine dioxide. Despite various claims to the contrary the major drawback of this process remains the need of using acetone in a pulp mill.

Rost and co-workers studied the potential of using an ozone treatment as an activating stage between two oxygen bleaching stages (*54*). The interstage ozone

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treatment resulted in lower kappa numbers as a function of the ozone charge. A second oxygen stage, after an OZ sequence, reduced the kappa number by approximately 5 units, independent of the ozone charge. The selectivity of the OZO bleaching sequence was found to be better when compared to the OO bleaching sequence. The efficiency of ozone was found to decrease with increasing pH and temperature. At low charges (0.2%) and at 50°C and pH of 4-5, ozone can be used as an activator between two oxygen stages without negatively affecting the selectivity of the process.

Li and co-workers compared the efficiency of peroxymonosulfuric acid (Px), peroxyacetic acid (Paa) and ozone (Z) as interstage activating agents for a two-stage oxygen delignification of softwood kraft pulp (*55*). The conditions of the Paa and Px stages were optimized. Under optimized conditions, the OPaaO sequence showed the best delignification efficiency and selectivity. The viscosity of the OPaaO and OPxO pulps were found to be superior to those obtained by OO and the OZO bleaching sequences. An examination of the molecular mass distributions of lignin dissolved in the acidic Paa, Px or Z stages, and of the lignin dissolved in the ensuing alkaline oxygen stages, demonstrated the depolymerizing effects of peroxyacids and ozone, which were held responsible for the lower kappa numbers obtained after the activated two-stage bleaching sequences.

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Nelson and co-workers studied the use of xylanase as an interstage treatment in a two-stage oxygen delignification process (*46*). The data showed that in the presence of the enzyme, at least 15% more lignin was removed, and the resulting pulps were of higher viscosity. The precise mechanism of the action of xylanase on the pulp fiber has not yet been fully elucidated. A number of propositions do exist, however, including that these enzymes act mainly on the removal of some alkali-resistant reprecipitated or reabsorbed xylans; thus promoting the extractability of lignin. (*56, 57, 58*)

Activation and Catalysis of Peroxide Delignification

Hydrogen peroxide has always been a technologically attractive oxidant to the pulp and paper industry. Due to its limited reactivity toward residual lignin, peroxide is unable to fully replace chlorine containing bleaching agents. Under alkaline conditions, high temperature accelerates the decomposition of hydrogen peroxide, which not only reduces the amount of peroxide available for bleaching (*59*), but also increases the amount of radicals formed, which have a detrimental effect on carbohydrates (*60*). Under acidic conditions, low pH and high temperatures could result in severe cellulose degradation due to the acid hydrolysis of cellulose.

Extensive investigations have been carried out on the stabilization of peroxide under conditions conventionally applied during the bleaching of chemical pulps. The effect of transition metals on peroxide decomposition, and the necessity of their efficient removal before peroxide treatment have been well documented (*61, 62, 63, 64, 65, 66*). Stabilized peroxide acts as a lignin-preserving bleaching agent, preferably removing chromophoric structures in residual lignin, but incapable of degrading the lignin network.

In efforts aimed to increase the reactivity of peroxide, harsher reaction conditions were employed (*67, 68, 69*). Higher temperatures and chemical charges increased the extent of delignification, but also promoted carbohydrate degradation, especially in the presence of transition metals.

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Pulp pretreatment prior to an alkaline peroxide stage offers another possibility for improving the overall selectivity of peroxide delignification. For example, softwood kraft pulp treatment with 3% nitrogen dioxide (on o.d. pulp) at 70°C and 40% consistency for 15 minutes, followed by washing and alkaline peroxide stage (1% H_2O_2 on the o.d. pulp) at the same temperature, removed about 60% of the residual lignin (*70*). It is believed that nitrogen dioxide pretreatment resulted in fragmentation of ether bonds of lignin, and the formation of new phenolic groups (*33*), which make the lignin more reactive towards the alkaline peroxide.

Lachenal and Papadopoulos proposed a highly efficient hydrogen peroxide delignification process for kraft pulps (*71*). It consists of an activation stage - pulp pretreatment by Cl_2, NO_2 or O_3, and a peroxide delignification stage at high temperature (90°C). In this manner, 50 to 80% delignification was attained using unbleached softwood and hardwood kraft pulps with only a 1% peroxide charge. After the peroxide stage, the degree of polymerization of the cellulose within the pretreated pulps (Cl_2, NO_2 or O_3) was found to be higher when compared to the peroxide bleached pulp without the pretreatment. The rationalization proposed to explain the increased delignification efficiency of peroxide was the formation of new phenolic hydroxyl groups possibly occurring *via* lignin demethylation reactions.

Pretreating kraft pulps with peracids (peracetic, Caro's, or mixed peracids) followed by a washing stage has been shown to increase the delignification effectiveness and brightness gains of an ensuing peroxide stage (*72, 73*). More specifically, the use of peroxyformic acid prior to a peroxide stage, has been described by Poppius and co-workers (*38*). Prebleaching pine kraft pulp with peroxyformic acid reduced the kappa number by 25-80%, depending on the amount of hydrogen peroxide applied, the concentration of formic acid and the temperature of the treatment. Such prebleached pulps attained a brightness of

80-85% in the subsequent alkaline peroxide bleaching sequence. The increased reactivity of the kraft residual lignin toward alkaline hydrogen peroxide was held responsible for the brightness gains observed (*74*).

Transition Metal Centred Peroxide Activators

Various approaches aimed at overcoming the low capacity of hydrogen peroxide to selectively degrade lignin structures have been centred around its activation. The addition of an activator or catalyst directly to the peroxide stage, which by interaction with the peroxide would generate a more potent oxidants *in-situ*, allowed improved delignification with minor or no carbohydrate degradation. In this respect, a number of peroxide activators of a variety of structures, involving different activation mechanisms have been examined.

The delignification of kraft pulps with hydrogen peroxide catalyzed by transition metals was initially proposed and patented by Eckert (*75*). An acidic peroxide delignification stage was shown to be improved by the addition of transition metals selected from the group of tungsten molybdenum, chromium, osmium, and selenium. As one of the examples, in the original patent, tungsten ion catalyzed acidic peroxide delignification of hardwood kraft pulp was compared with alkaline peroxide bleaching. While the brightness gains and pulp viscosities were similar, the degree of delignification for the tungsten assisted acidic peroxide delignification stage was reported to be higher. The rationale behind the effectiveness of these systems was that the transition metal oxides, under acidic conditions reacted with peroxide to form transition metal peroxo-complexes, which are stronger oxidants than peroxide itself.

The ability of molybdate metal oxides to catalyze the acidic peroxide delignification of kraft pulps was confirmed a few years later by Kubelka *et al.* (76). The catalytic effect of molybdate was attributed to its ability to form, under acidic conditions, reactive diperoxo complexes with peroxide (*77*).

The data of Kubelka co-workers showed that under acidic conditions (pH 5) and a 2% peroxide charge, the addition of 500 ppm of sodium molybdate reduced the kappa number by approximately 10 units. Acidic peroxide alone, in the absence of molybdate reduced the kappa number of the same pulp by only 3 units. Unfortunately, however, the pronounced delignification observed, was accompanied by serious viscosity losses (*76*). On the same topic, Agnemo used ammonium molybdate to activate peroxide delignification (*7*). Using only peroxide at pH ~ 5, Agnemo showed that no delignification was possible, however, addition of molybdate ions resulted in significant delignification.

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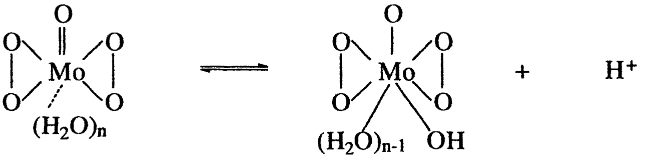

Scheme 4: Diperoxo molybdenum complexes formed from the interaction of molybdenum ions and acidic peroxide (77).

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In 1995, Jäkärä and co-workers studied the effect of silicomolybdate activated peroxide for ECF and TCF bleaching sequences (*78*). Under acidic conditions, silicomolybdate ions were invoked to react with peroxide to form silicoperoxomolybdates. Such ions, under optimal conditions are anticipated to be selective delignifying agents. Mill trials with such systems showed that 40-60% delignification could be obtained without any adverse effects on pulp quality. A fully bleached (89% ISO) softwood kraft pulp was obtained, both in the laboratory and during the mill-scale trials, by using only oxygen and activated peroxide as the bleaching agent. Jäkärä and co-workers compared the delignification efficiency of silicomolybdate activated hydrogen peroxide with peracetic acid (distilled and equilibrium) (*79*). Under optimum conditions, similar kappa number reductions and viscosity values were obtained. Both, peracetic acid (2.5% charge) and molybdate (450 ppm, calculated as Mo) were found to activate hydrogen peroxide (1.5% charge) and reduce the kappa number of softwood kraft pulp from an initial value of 16 to approximately 9. While the molybdate activated peroxide systems offered somewhat lower brightening efficiencies compared to peracetic acid, they were claimed to offer the possibility of regeneration *via* filtrate recirculation.

In an effort to demonstrate that transition metal peroxo-complexes were responsible for the activation of peroxide, Suchy and Argyropoulos studied the effect of a transition metal peroxo complex on an alkaline peroxide delignification (*80*). Ammonium triperoxo-phenanthroline vanadate was initially synthesized and then added to the alkaline peroxide stage. At 0.5% activator charge, the kappa number of oxygen delignified pine kraft pulp was reduced from 16.2 to 6.3 (61% delignification) using a single alkaline peroxide stage. At the same time, the brightness of the pulp was increased by 5.7% ISO when compared to an alkaline peroxide bleaching stage at the same conditions in the absence of the activator.

Recently, tungstate, molybdate and vanadate based polyoxometalates have been identified as activators of acidic peroxide delignification (*81*, *82*). Bianchi and co-workers investigated various types of heteropolyacids (HPA) of Keggin type as potential catalyst of acidic peroxide delignification (*81*). Using 0.5 mmol/L $H_3PMo_{12}O_{40}$ and 0.1% H_2O_2 an 70°C for 4 hours, a hardwood kraft pulp with a kappa number of 2.1 and viscosity higher than control trials under the same conditions without employing a HPA as a catalyst, can be achieved. However, the use of HPAs as catalysts was found not effective for softwood pulp delignification. Evtuguin and co-workers have examined the potential of heptamolybdopentavanadophopsphate heteropolyanion - HPA-5 toward activating an acidic peroxide delignification stage (*82*). Despite an excellent delignification efficiency demonstrated by the system (from kappa 15.9 to 6), a significant reduction in pulp viscosity was also apparent. The latter was most likely caused by the extensive decomposition of hydrogen peroxide and hydroxyl radical attack of the cellulose.

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Metal oxides such as tungstate, molybdate, methyltrioxorhenium, as well as some tungstate and molybdate based polyoxometalates, were evaluated as potential catalysts of acidic peroxide delignification (AP) by Chen and co-workers (*83*). The catalyzed acidic peroxide stage increased the degree of delignification of oxygen delignified softwood kraft pulp with increased selectivity compared to the non-catalyzed control bleaching. Simple metal oxides (tungstate, molybdate) were found to be equally as effective as their polyoxometalate counterparts in catalyzing acidic peroxide bleaching. Methyltrioxorhenium was found to be inactive in acidic peroxide bleaching of kraft pulps.

Patt demonstrated improved delignification efficiency of peroxide catalyzed by metal complexes. These catalysts were single or multiple nuclear metal complexes of manganese or iron, which can be present in the oxidation states II, III, IV, or V or in mixture of these states. Other parts of the complexes were various ligands (*84*). Catalyzed P-stage resulted in improved delignification and selectivity of the peroxide bleaching stage. The metal complexes were also tested in peroxide reinforced oxygen delignification and were shown to be capable of increasing the delignification rate of the process.

A bi-nuclear manganese complex in which the manganese ions are present in the +IV and +III oxidation states, linked *via* an acetate and two oxygen bridges (Scheme 5) was shown to selectively improve peroxide delignification.

Patt and Mielisch and Patt and co-workers using an OQ(OP)P bleaching sequence demonstrated the effectiveness of the proposed system for the production of a fully bleached TCF softwood kraft pulp. Amongst the various parameters examined, the authors showed that this system may activate peroxide at the relatively low temperature of 50°C, while higher temperatures offered significantly reduced retention times. The proposed catalyst was shown that it could be applied either in one or two P stages. When applied at a level of 20ppm, during the OP stage, a brightness of 88% ISO was obtained. When the

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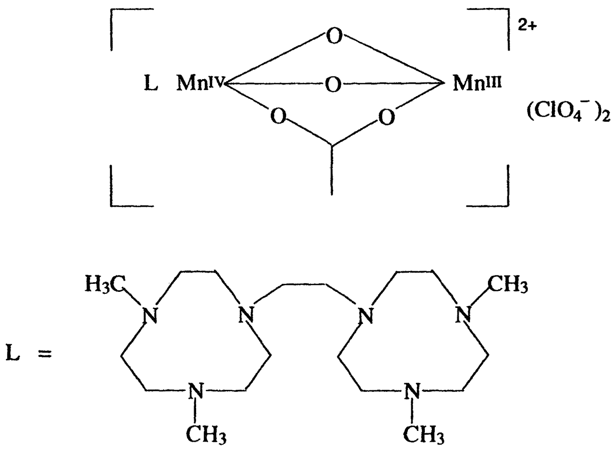
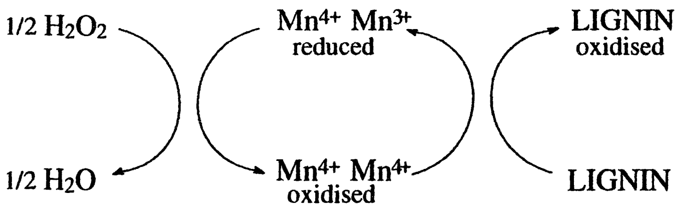

L
Mn^IV
O
O
O
O
Mn^III
2+
(ClO4⁻)2
L =
H3C
N
N
N
N
CH3
N
N
CH3
CH3

Scheme 5: Chemical structure of the nucleus and the ligands of the selected manganese based catalytic activating peroxide system. (85)



Scheme 6: Proposed redox cycle of a catalyst with two manganese nuclei. The oxidized complex is oxidizing lignin, whereby it is reduced, and then the cycle is reinitiated. (85)

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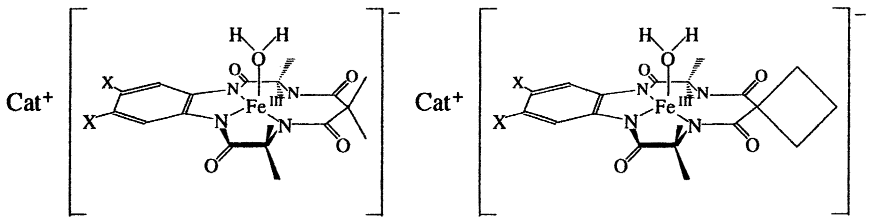
catalyst was also applied in the ensuing P-stage, a brightness of 90% ISO was obtained at elevated temperatures (*85, 86*).

Cui and co-workers investigated the reactivity of lignin toward hydrogen peroxide catalyzed by binuclear manganese complex, $[LMn(IV)(\mu\text{-}O)_3Mn(IV)]^{2+}$(abbreviated as Mn(IV)-$Me_4$DTNE) where L stand for 1,2-bis-(4,7-dimethyl-1,4,7-triazacyclonon-1-yl)ethane (*87*). Based on the model compounds reaction studies, it was suggested, that hydrogen peroxide catalyzed by Mn(IV)-Me_4DTNE would be capable of oxidizing the α-hydroxyl groups and conjugated C-C double bonds in the residual lignin of chemical pulps. The resulting carbonyls and epoxides would be then susceptible to nucleophilic attack leading to further degradation and fragmentation of the residual lignin (*87, 88*). Also examined was the efficiency of Mn(IV)-Me_4DTNE catalyzed hydrogen peroxide in the delignification of a pine kraft pulp. More specifically, an addition of Mn(IV)-Me_4DTNE (60 ppm on o.d. pulp) to the peroxide bleaching stage at 80°C with a charge of 4% peroxide at 10% consistency for 120 min significantly improved the degree of delignification from 25 to 46%, compared to the non catalyzed delignification under the same reaction conditions (*88*).

Recently, Collins and co-workers developed a new class of peroxide activators (*89*). The research of this team was focused at resolving the incompatibility issue that most ligands have with strongly oxidizing metal ions or media. Consequently, the team has proposed a new class of iron (III) complexes involving macrocyclic tetraamides. These complexes were shown to catalytically activate aqueous hydrogen peroxide offering a variety of oxidation reactions.



1a; X = Cl
1b; X = H
1c; X = OCH_3

Cat^+ = Li^+, $[Me_4N]^+$, $[Et_4N]^+$, $[PPh_4]^+$

2a; X = Cl
2b; X = H

Cat^+ = Li^+, $[Me_4N]^+$, $[Et_4N]^+$, $[PPh_4]^+$

Scheme 7: Iron based macrocyclic tetraamide activators of hydrogen peroxide (89)

Using softwood kraft pulp, Collins *et al.* demonstrated that the aforementioned systems could catalytically activate peroxide rapidly bleaching kraft pulp under mild conditions in basic aqueous media. Micromolar concentrations of the proposed compounds, at temperatures ranging from ambient to 90°C were claimed to selectively direct the oxidation ability of peroxide toward the lignin. More specifically, a one hour peroxide bleaching stage at 90°C with a charge of 4% peroxide (on o.d. pulp) and 80 ppm of activator reduced the kappa number of a softwood kraft pulp from 21.5 to 7.8, while its viscosity was maintained at an acceptable level.

Nitrogen Centred Peroxide Activators

The literature contains a number of accounts in which various peroxide activators containing nitrogen have been examined. Amongst them cyanamide and its dimer, dicyandiamide occupy a prominent position. The cyanamide activated peroxide bleaching process of chemical pulps was initially proposed and patented by Hammer *et al.* and Sturm (*90*, *91*). Alkaline hydrogen peroxide was thought to react with cyanamide to form a peroxyimidic acid intermediate. Because such a species would be expected to have a higher oxidation potential than peroxide, its effectiveness should be greater (*92*).

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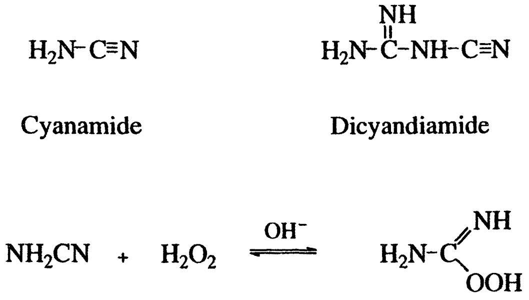


Scheme 8: Showing the interaction of cyanamide with alkaline hydrogen peroxide, generating peroxyimidic acid (92).

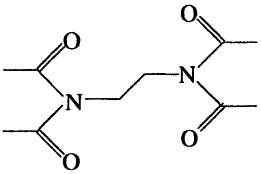
When compared to control runs, cyanamide activated alkaline peroxide bleaching stages produced pulps of higher brightness (by nearly 10 ISO units) with comparable viscosities (*90*, *91*).

Research by Chen, showed that dicyanediamide, the dimeric form of cyanamide, is a more effective peroxide activator allowing oxygen delignified kraft pulps to be efficiently bleached (*93*), most likely proceeding with a mechanism similar to that of cyanamide.

In contrast to the proposition of Sturm and Kuchler (*92*), recent work by Kadla and co-workers, on the reactions of lignin and lignin model compounds with cyanamide activated peroxide, demonstrated the involvement of superoxide anion radicals during the reaction (*94*). The data provided evidence for two competing reaction systems, radical and ionic, in which the majority of the chemistry proceeds via a radical mechanism. The reactions were found to be strongly dependent on pH, with optimum reactivity at pH 9-10 when equimolar amounts of cyanamide and peroxide are used. Further examination of the interaction of cyanamide with alkaline hydrogen peroxide confirmed the presence of a radical intermediate, perhydroxyl radical from peroxide and the corresponding radical cation emerging from cyanamide (*95*). Four main reaction pathways were thus invoked; aromatic hydroxylation, oxidative demethylation, oxidative coupling and aromatic ring opening with subsequent fragmentation reactions. Oxidation reactions involving radical intermediates were thought to predominate over radical coupling reactions.

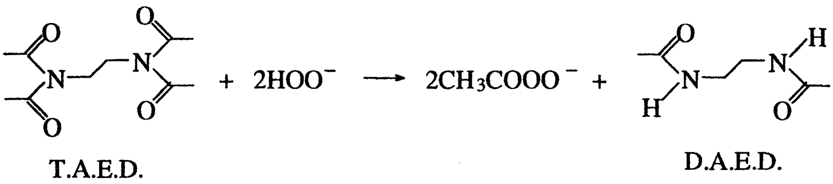
Kang and co-workers have examined the effect of adding cyanamide and molybdate ions in a neutral peroxide delignification of oxygen delignified softwood kraft pulp (*96*). A neutral peroxide stage in the absence of an activator produced only 4.1% delignification, while the addition of 2% of cyanamide increased the delignification to 23.5%, while the addition of 500 ppm of Mo^{6+} ions increased the delignification to 27.9%. The simultaneous addition of cyanamide and Mo^{6+} further improved the delignification to 36.9% with no decrease in the viscosity of the resulting pulp. The effect of other metal additives (W^{6+}, V^{5+}) in the cyanamide/metal activated peroxide system were also studied and compared. W^{6+} showed similar results to those of Mo^{6+}, while V^{5+} was found to be totally ineffective.

Tetra acetyl ethylene diamine (TAED) was shown to increase the brightening potential of peroxide by generating peracetic acid *in-situ* as a result of the interaction of TAED with peroxide (*97*).



Scheme 9: Tetra Acetyl Ethylene Diamine (TAED). (97)

Depending on the pH of the bleaching stage, TAED is thought to react with peroxide to produce peracetic acid or peracetate anion. Both species are known to be stronger oxidants than hydrogen peroxide. In addition, generating peracetic acid *in-situ* avoids handling and capital investment complexities involved when using peracetic acids solutions.



Scheme 10: The reaction of TAED with hydrogen peroxide under alkaline conditions. (97)

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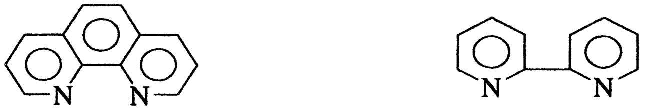
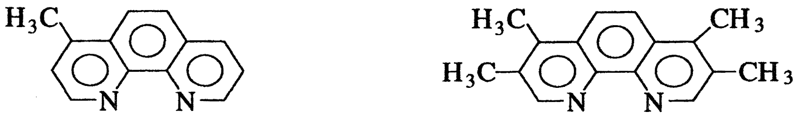
Under alkaline conditions, (pH 9-10) TAED reacts with peroxide rapidly. For example, at 20°C the reaction is complete within 10-15min. At pH's higher than 10 the reaction rate increases but the stability of the peracetate anion and the rate of side reactions, involving hydroxide and TAED also increase. In general, the optimum pH of TAED assisted alkaline peroxide delignification is lower than that used in conventional alkaline peroxide stages. TAED also reacts with peroxide under neutral and even acidic conditions while under such conditions peroxide alone is practically ineffective as a delignifying agent. In general, Croud and Mathews state, that this system offers an environmentally benign way of activating peroxide by decreasing the bleaching reaction time without the need of silicate stabilizers, providing possible chemical and cost savings. In addition, Turner and Mathews claim that the use of TAED can readily and safely be incorporated into ECF and TCF bleaching sequences for a number of pulp types (*98*).

Nitrogen heterocyclic compounds, containing an α,α'-diimine group as part of the aromatic system, is another potential set of peroxide activators. Such a group is that of polypyridines. Jaschinski and Patt in 1998 showed that delignification and brightness of chelated oxygen delignified softwood kraft pulps, can be significantly improved by adding polypyridines into an alkaline peroxide stage (*5*).



1,10-phenanthroline 2,2'-bipyridine



4-methyl-1,10-phenanthroline 2,4,7,8-tetramethyl-1,10-phenanthroline

Scheme 11: Polypyridines containing an α,α'-diimine group as part of an aromatic system applied in various bleaching trials. (5)

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Charges of 4-methyl-1,10-phenenanthroline as low as 0.025%, showed significant brightness gains (11% ISO) when compared to control experiments, with minimum viscosity losses. A significant brightness improvement was also observed when a peroxide stage was carried out at high temperatures. In general the brightness gains were found to be dependent on the dose of polypyridine applied. Amongst the compounds examined, 4-methyl-1,10-phenanthroline 1,10-phenanthroline and 2,2'-bipyridine were found to be the most effective.

Peracids

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Peracids, which are essentially derivatives of peroxides, are substantially stronger oxidizing agents than alkaline oxygen or peroxide. From this point view, bleaching with peracids can be considered as a form of activated peroxide delignification. The oxidizing power of peracids is similar to that of chlorine dioxide and chlorine and as such their delignifying and bleaching potential should be similar to chlorine and chlorine dioxide, with the obvious advantage of being chlorine free.

The ability of peracids to delignify under neutral and/or acidic conditions, coupled with environmental pressures for replacing chlorine and chlorine containing bleaching agents, makes these compounds promising alternative oxidizing agents for chemical pulp bleaching. Peracids have been examined as potential agents for replacing an initial chlorination stage or immediately following an oxygen delignification stage. They have even been applied in the final brightening stages of a bleaching sequence and as activators prior to or between two oxygen or peroxide stages (*99*). Their proven versatility, therefore, dictates that a close attention be paid to them in this review.

Peracetic Acid

The potential of using peracetic acid for chemical pulp delignification was realized as early as the 1950's (*100*). However, its high cost coupled with its transportation complexities prevented its wider industrial utilization. In 1966, Bailey and Dence described the use of peracetic acid as a brightening and a delignification agent of chemical pulp. These authors compared the Kappa number and the optical and strength properties of the emerging pulps with those obtained from conventional bleaching and delignifying reagents (*101*). Despite the fact that peracetic acid showed great promise as a delignification agent its cost and poor selectivity prohibited its industrial applicability. The peracetic acid used in these early studies was an equilibrium product prepared by mixing appropriate amounts of acetic acid and peroxide in the presence of sulfuric acid as a catalyst.

$$CH_3COOH + H_2O_2 \overset{H_2SO_4}{\rightleftharpoons} CH_3COOOH + H_2O \qquad (3)$$

As implied by the above equilibrium and under conditions that promote it, peracetic acid typically contains high concentrations of acetic acid and hydrogen peroxide. The low amounts of peracid present in the equilibrium mixture severely affected the economics of the process and in addition, the peroxide present in the mixture together with transition metal ions impurities of the pulp may cause serious viscosity losses (*102*).

In an effort to reduce the chemical costs associated with the process, Christiansen and co-workers investigated the possibility of *in situ* generating peracetic acid during a bleaching stage (*103*). In their efforts peracetic acid was formed *in situ* by reacting hydrogen peroxide and acetic anhydride. Bleaching kraft pulp with peracetic acid formed *in situ,* under optimum conditions, resulted in brightness levels similar to those obtained by chlorine dioxide at the same oxidation equivalent. However, the strength of the pulp was significantly lower and its production costs significantly higher when compared to the chlorine dioxide bleached pulp.

Several approaches aimed at improving the selectivity of peracetic acid have been suggested. Hill and co-workers focused their efforts on transition metal control, aimed at reducing the radical decomposition of the unreacted peroxide (*104*) and of peracetic acid (*105*). Other efforts involved the removal of peroxide from the peracetic acid equilibrium mixture by distillation (*104*). Distilled peracetic acid can actually be prepared by vacuum distillation of a mixture of acetic acid, hydrogen peroxide and sulfuric acid (*106*, *107*). This process offers a high conversion of peroxide to peracid and the excess acetic acid, peroxide and sulfuric acid are removed. As such, the peroxide responsible for cellulose damage is eliminated and the cost of the peracetic acid is reduced since the excess chemicals may now be recycled.

Jäkärä and co-workers have compared the delignification efficiencies of equilibrium (e)Paa with distilled (d)Paa delignification stages in TCF bleaching sequences of softwood kraft pulp (*79*). It was thus confirmed that different optimal initial pH's for ePaa (pH 4.5) and dPaa (pH 5-7) were required. The delignification efficiency of the ePaa and the dPaa stages showed little difference. However, as anticipated, considerable differences were observed in the total amount of chemical required.

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Vuorenvirta and co-workers recently compared the efficiency of using equilibrium and distilled peracetic acids in TCF softwood pulp bleaching (*108*). Their data confirmed the fact that equilibrium and distilled peracetic acids have different optimum pH operating ranges. The optimum initial pH for ePaa proved to be 4-5, and for dPaa 5-8. However, the delignification achieved with distilled and equilibrium peracetic acids were the same when the bleaching was carried out under optimized pH conditions. These authors also investigated the possibility of reducing the consumption of Paa by applying an acid stage prior to bleaching. Their data showed that the final kappa numbers of the acid washed and chelated pulp were about the same. However, there was a significant drop in chemical consumption if the reaction time of the acid stage was long enough (120 min).

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Jääskeläinen and Poppius-Levlin studied the factors affecting the kinetics of oxygen-treated pine kraft pulp delignification with peroxyacetic acid (*109*). It was confirmed that equilibrium and distilled Paa gave equally high delignification rates and pulp viscosity, while ePaa led to slightly better pulp brightness, most likely due to the presence of peroxide in the ePaa mixture. Jääskeläinen and Poppius-Levlin concluded that the most important factors affecting the kinetics of delignification in the Paa stage were Paa concentration, temperature and pH.

A full-scale mill trial using peracetic acid was carried out in Sweden in 1994 and in 1995, the first mill in the world started to use peracetic acid for TCF pulp production (*99*). Selective delignification, good pulp strengths and low investment costs were amongst the advantages claimed for this process when compared to other TCF sequences. However, production costs were higher.

The possibility of using distilled peracetic acid was examined in various mill-scale trials in Finland (*110*). This effort lead to the conclusion that peracetic acid could be used for both brightening and delignifying purposes. In ECF bleaching, the advantage of Paa is a high brightness level and the reduced need for active chlorine in bleaching. The use of Paa in TCF bleaching resulted in a pulp with low kappa number and high brightness with good stability. Various economic disadvantages were still obvious.

Caro's Acid and Other Peracids

Growing interests in non-chlorine bleaching agents have promoted attention to peroxy acids, other than peracetic acid, as possible delignification and bleaching agents. Peroxymonosulfuric acid, its salts and more recently performic acid, have all been reported as effective oxidizing agents for delignifying and bleaching chemical pulps (*38*; *72, 111, 112*).

$$H_2SO_4 + H_2O_2 \rightleftharpoons H_2SO_5 + H_2O \qquad (4)$$

The conversion of peroxide to Caro's acid is affected by the amount of water present in the reaction mixture. Its concentration reaches a maximum at about 45% conversion, when an excess of 99% sulfuric acid and 50% hydrogen peroxide (1.5:1.0 mole ratio) is used. The conversion of the process can be increased by minimizing the amount of water or by using more concentrated peroxide (70%) and sulfuric acid (oleum) (*113*). Both of these routes, however, have aroused serious safety concerns (*114*). Despite these, mill trials using Caro's acid have been conducted yielding high brightness softwood and hardwood kraft pulps (*115*).

Geng and co-workers have described another means of increasing the peroxide conversion to the peracid (*72*). By adding acetic acid to the equilibrium mixture of Caro's acid a mixture of peracetic and peroxymonosulfuric acids is formed. This permits the high conversion of peroxide to the peracids without using excessively high peroxide and sulfuric acid concentrations.

$$H_2SO_4 + H_2O_2 \rightleftharpoons H_2SO_5 + H_2O_2 + CH_3COOH + H_2O_2 \rightleftharpoons CH_3COOOH + H_2O \quad (5)$$

The work of Song and co-workers has essentially confirmed the effort of Geng *et al.* since they reported that by adding one mole of acetic acid to the Caro's acid mixture increased the conversion of peroxide to peracids to a 94% level (*111*). The role and possible application of Caro's acid (Px) and mixed peracids (Pxa) in ECF and TCF bleaching sequences were also investigated in the following efforts (*72, 111, 116*).

Devenyns and co-workers have shown that they were able to obtain fully bleached softwood kraft pulp by using a peroxygen based bleaching sequence (*73*). Oxygen delignified and chelated pulp was prebleached with peroxide followed by a peracid stage (peracetic, Paa and Caro's Px acids). The final brightening was obtained by applying a P* stage (alkaline hydrogen peroxide at high consistency with particular attention being paid to transition metal removal and stabilization). Using the described OQPPaaP* and OQPPxP* sequences, fully bleached TCF kraft pulp was produced with adequate mechanical properties.

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A TCF bleaching sequence composed of an acid pretreatment, acidic peroxymonosulfate, oxygen delignification and alkaline peroxymonosulfate brightening was described by Springer and McSweeny (*112*). Under the optimal bleaching conditions, a pine kraft pulp with a final brightness of 83% ISO was obtained while when the same sequence was applied to an aspen kraft pulp a final brightness of of 86% ISO was obtained.

Amini and Webster compared the delignification efficiency of mixed peracids (Pxa) with oxygen (*114*). A comparison of oxygen delignification and mixed peracids stages followed by Eop, showed that both reduced the kappa number of pine kraft pulp (kappa 34) by half. The PxaEop stage, however, gave in higher brightness. Experiments with eucalyptus kraft pulp (kappa number 20) showed an even better performance for peracids. A single Pxa stage was shown to have a higher delignification efficiency than a single oxygen delignification stage. The combination of Pxa and Eop stages resulted in 60% delignification. This is substantially more than the delignification obtained after the single oxygen. When a PxaEop sequence was applied after an oxygen stage, the kappa number was reduced from 12 (after the oxygen stage) to 5.1, and the brightness reached 69% ISO. By comparing the delignifying and brightening efficiencies of peracetic acid, Caro's acid and mixed peracids, it was concluded that for a variety of conditions, equal amounts of peracid (equal active oxygen in its peracid form, or equal molar amounts of peracid) gives similar delignifying and brightening performance.

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Another comparison of peracetic acid and Caro's acid has been reported by Basta and co-workers (*107*). When oxygen delignified softwood kraft pulp was bleached with a sequence composed of a peracid treatment, followed by a chelating stage and a final alkaline peroxide stage, superior selectivity was shown when peracids (for peracetic acid both dPaa and ePaa were used) compared to Caro's acid. A similar comparison with ozone in a QPZP bleaching sequence proved once again the higher selectivity of dPaa.

Troughton and co-workers examined the effect of inserting a peracid stage into a TCF bleach sequence, consisting of a metal controlling Q-stage, followed by several alkaline peroxide stages (*117*). By inserting a peracid stage into the QPPP sequence it was found that the pulp brightness may be increased by 5-10 ISO points without pulp strength losses. When the delignification efficiencies for the various sequences were compared, the effect of the peracid stage was found to be due to a more extended delignification. For example, when a softwood oxygen delignified kraft pulp was bleached by a QPPxP sequence its kappa number was reduced to 2.4. However, a somewhat better delignification was obtained when Caro's acid was replaced by peracetic acid. The final kappa number, after the QPPaaP sequence was 2.1. Equivalent bleaching, using only peroxide (QPP) was found to reduce the kappa number to only 5.7.

Liebergott showed that by applying a peracetic acid pretreatment on an unbleached softwood kraft pulp, followed by an Eop stage can increase the delignification from 35% (QEop) to 48.9% (PaEop) (*8*). Similar results were also observed when an oxygen delignified softwood kraft pulp was used. The Pa treatment increased the extent of delignification after an Eop stage from 20% (for an OQEop sequence) to 40% (for an OPaEop sequence). The kappa number was reduced from 16.4 (after oxygen delignification) to 9.8.

Poppius and co-workers reported the use of peroxyformic acid in TCF bleaching (*38*). The degree of delignification of a pine kraft pulp achieved with peroxyformic acid and hydrogen peroxide was 25-80%, depending on the concentration of formic acid, amount of peroxide and the temperature of the treatment. Subsequent alkaline peroxide bleaching stages raised the brightness to 80-85% ISO. In addition, the same team showed that peroxyformic acid may also increase the efficiency of an oxygen delignification stage by ensuring a substantial decrease in the incoming kappa number prior to the oxygen stage (*74*). Kappa numbers as low as 4-6 were obtained for pine and birch kraft pulps after the (PFA)O sequence. Consequently, brightness values of 90% ISO were reached for both the pine and birch kraft pulps predelignified with peroxyformic acid and bleached with a sequence using oxygen, ozone and alkaline peroxide.

Springer examined peroxymonophosphoric acid as a possible delignification agent for wood and kraft pulp (*118*). Pine kraft pulp was delignified with a dilute aqueous solution of peroxymonophosphoric acid and its delignification selectivity was compared with peroxymonosulfuric acid. This exploratory study showed that, under identical reaction conditions, peroxymonophosphoric acid delignified more rapidly and more selectively than peroxymonosulfuric acid. However, the incompatibility of the phosphates with current mill recovery systems is a major drawback.

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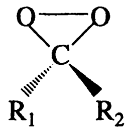
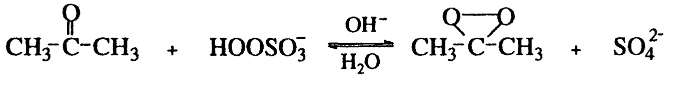
Dioxiranes

Dioxiranes have recently been identified as a novel and selective class of non-chlorine bleaching agents for chemical pulps (*119*). This is a class of powerful electrophilic oxidants efficiently transferring oxygen onto aromatic and/or unsaturated substrates. The major feature of such compounds is their effectiveness to transfer a single activated oxygen atom onto an aromatic substrate. Consequently, this class of oxidants was termed "*Activated Oxygen*" (AO), in reference to its use in a bleaching sequence. Dimethyldioxirane (DMD) represents the simplest member of this series. This cyclic organic peroxide can be generated by the interaction of peroxymonosulfuric (Caro's) acid with acetone (*120*). The potential of DMD for pulp bleaching purposes was initially recognized, patented and reported by Lee and co-workers at PAPRICAN and latter was confirmed by Ragauskas (*121*; *122, 123*).



Scheme 12. Chemical structures of dioxiranes. R_1 and R_2 can be the same or different, and may be linked to form cyclic compounds. (119)

Lee and co-workers initially studied the bleaching efficiency of isolated DMD (*121*) and latter of *in-situ* generated DMD (*122*). A one stage treatment of a hardwood kraft pulp with isolated DMD (0.55% as AO, calculated as 1 activated oxygen per molecule of DMD, or 2.5% isolated DMD) for one hour, reduced the kappa number from 16.4 to 3.4 (79% delignification) (*124*). The yield of activated oxygen, however, in the isolated form, based on the charge of monoperoxysulfate was found to be extremely low, seriously precluding the potential of the process for industrial application. Bleaching with *in-situ* generated AO was carried out by mixing the pulp slurry with acetone in an aqueous slurry. Sodium bicarbonate was added to control the pH (7-7.5) and then monoperoxysulfate was added to the pulp slurry. The reaction of acetone with monoperoxysulfate at a near neutral pH, generated AO *in-situ*, which subsequently bleached the pulp.



Scheme 13: Synthesis of dimethyldioxirane (DMD) from acetone and peroxymonosulfuric acid. (119)

Delignification of softwood kraft pulp with *in situ* generated DMD (2.7% AO, 12.5% DMD) followed by an alkaline extraction stage, reduced the kappa number from 31.5 to 5.4 (83% delignification). Lee's data were latter confirmed by Ragauskas who bleached a softwood kraft pulp with a 5% isolated DMD charge (*123*). The initial kappa number of 39.5 was reduced to 12.5 (68%

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delignification). Both groups defined DMD as a very selective delignification agent either in its isolated form or generated *in situ* within the pulp slurry.

Further work was carried out latter by McDonough and co-workers who studied the efficiency of DMD in bleaching oxygen delignified kraft pulp, and its effects in a TCF bleaching sequence using with oxygen and peroxide (*125*). Oxygen delignified softwood kraft pulp was sown to be effectively delignified by treating it with a 4% DMD charge. The kappa number was reduced from 14.1 to 5.8. Amongst various TCF bleaching sequences examined, O(AO)QP was found to be the most successful. When a DMD stage was added at the end of the bleaching sequence (OQP(AO)) the final brightness and viscosity were lower. DMD was not found to be an ineffective brightening agent because it introduces chromophores into the remaining lignin. It was found, however, to have a positive effect on the brightening power of a subsequent peroxide stage. DMD charges of up to 2% had virtually no effect on viscosity while charges of 4% and above resulted in noticeable viscosity losses. This effect was rationalized on the basis that, at low lignin contents, the excess DMD was not consumed, increasing the possibility of it oxidatively attacking the cellulose.

Lee and co-workers also examined the efficiency of DMD in the bleaching of oxygen delignified kraft pulp (*119*). A DMD treatment (AO charge 2.5%) of an oxygen delignified softwood kraft pulp followed by oxygen-peroxide-reinforced extraction (Eop) reduced the kappa number from 12.6 to 2.0. Viscosity and strength properties were the same as those observed after an O(C+D)Eop sequence. Consequently, since AO was shown to delignify kraft pulps to kappa numbers as low as 3 or less, it was thought that it would be possible to produce fully bleached TCF softwood kraft pulp by a single peroxide brightening stage preceded by a chelating treatment (Q). An OAEopQP bleaching sequence using a 2.5% AO charge was shown to yield a pulp of 90.9% ISO brightness with a severe viscosity loss at the peroxide stage.

In an effort to shed more light on the effectiveness of dioxiranes toward their demonstrated bleaching efficiency, Argyropoulos and co-workers have studied the reactions of DMD with lignin model compounds (*126*). DMD was found to electrophilically oxidize the aromatic rings of both etherified and non-etherified softwood lignin model compounds. This is an advantage that DMD has over other bleaching agents (ClO_2, H_2O_2, O_2, etc.) since other agents require free phenolic hydroxyl groups to act upon. On the same front, Bouchard and co-workers studied the stability of DMD under kraft pulp bleaching conditions (*127*). This work showed that the decomposition of DMD was accelerated by the presence of transition metals. However, the fast reaction rate of DMD with the pulp was found to partially counteract the deteriorating effects of such metals.

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Concluding Remarks

Exploratory attempts to improve the delignification and bleaching efficiency of oxygen and/or hydrogen peroxide commenced almost as soon as their delignification and brightening potential was realized. During the early efforts, the imposing limitations were economic and safety issues associated with the improvements of a particular process. During the past decade, however, environmental issues and regulatory forces have seriously altered the way we view such improvements. Despite the renewed interest in developments in this area and our new outlook, many processes, activators and catalysts, that have showed significant potential for application in oxygen or peroxide delignification fail to see widespread industrial applicability since the same economic and safety considerations still apply. More specifically, while peracetic acid and various other agents have been documented to efficiently delignify and bleach pulps, their high cost, relative instability and sometimes their incompatibility with the existing energy recovery systems, still prevents their widespread industrial utilization. Alternatively, the potential of catalytically activating hydrogen peroxide still needs to be fully explored and realized. Promising research endeavors in this area are already underway, using novel classes of compounds and emerging principles of organic and bioinorganic chemistry. Perhaps some of the answers may lie in the way nature reduces oxygen to water, controlling the process using various enzymatic receptors. Understanding the principles of biomimetic oxidations and applying them to our industry could conceivably offer some trully revolutionary processes that may guide our industry into the new millenium.

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Chapter 2

A Review of Lignin Model Compound Reactions under Oxygen Bleaching Conditions

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Associated with the environmentally driven increase in the use of oxygen bleaching technologies, has been an increased emphasis on understanding the mechanisms involved in this process through model compound studies. In this chapter, these studies will be reviewed from a standpoint of how the model compound structures relate to residual lignin, how functional groups and linkages affect reaction kinetics, the nature of the oxygen compounds involved in the reaction, and the pathways involved in degradation.

Investigations into the reactions involved in oxygen delignification have often focused on structural changes to residual lignin after bleaching. More often, however, studies have centered on reactions of lignin model compounds. There have been a significant number of these studies undertaken in the last three decades and therefore an overwhelming amount of results to interpret. It is important when reviewing these results to note that many of the model compounds studied do not represent structures of importance in residual lignin and that the rates of many of the reported reactions are to slow to be considered

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of any importance. In this chapter, the effects that functional groups and linkages have on oxidation rates will be reviewed noting which results are of importance. Additionally, information will be provided on the current knowledge of the reaction mechanisms involved in oxygen delignification and the radical and anionic oxygen species responsible.

Reactivity of Model Compounds

In many of the investigations directed towards understanding the mechanisms involved in oxygen delignification, model compounds containing the linkages and functional groups assumed to represent those found in residual lignin or formed during bleaching have been studied. Generally, the main goal of this research has been to identify the lignin structures that are stable or only slowly react under oxygen bleaching conditions. These are the structures that are assumed to be responsible for limiting the extent to which oxygen delignification can be used.

The selection of functional groups and linkages to investigate has been based upon the presumed structure of residual lignin. These theoretical models have been developed using the results of wet chemical procedures such as permanganate oxidation (*1*) and acidolysis (*2*) as well as ^{13}C NMR (*3*, *4*) and ^{31}P NMR (*5*) procedures. The wet chemical techniques can be applied directly to pulp samples although information is only obtained from those structures containing free phenolic hydroxyl groups. Only a small portion of the lignin is therefore analyzed. The NMR analysis requires that the residual lignin be isolated using methods such as a mild acidolysis (*6*) or enzymatic hydrolysis (*7*). These procedures can introduce structural changes in the lignin although evidence has been obtained (*8*) using a flow through reactor that the dioxane acidolysis isolation procedure does not significantly alter lignin composition. An excellent review of the procedures used in the isolation and characterization of residual lignin has recently been published (*9*).

Unfortunately, a complete understanding of the structural elements in residual lignin has yet to be obtained. Therefore, although the model compounds studied to date have been representative of structures in residual lignin, they may or may not represent the structures responsible for limiting delignification.

Monomeric Model Compound Reactivity: Effect of Substituents

Early studies on lignin oxidation focused on easily obtainable simple phenolic model compounds such as creosol and vanillin. These types of compounds continue to be used to test the effects of substituents on ring

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reactions. Information on the frequency of ring substituents in residual lignin is presented in Table I. The reactivity of model compounds containing these groups under oxygen bleaching conditions is presented in Table II.

Phenolic Hydroxyl

The content of free phenolic hydroxyl groups in native lignin is very important in terms of lignin solubility and reactivity. Determination of the amount of these groups has been accomplished through a variety of analytical methods with values of between 20 to 30 groups/100 phenylpropane units reported (*10*). Cleavage of ether linkages during pulping significantly increases the number of free phenolic hydroxyl groups in dissolved kraft lignins to levels as high as 60-65/100 C9 units (*11*). In residual lignin, however, the level increases only slightly to 25-35 groups/100 phenylpropane units (*3*, *12*, *13*).

Table I. Functional Group Content in Residual Lignin

Functional Group	*Amount Relative to Native Lignin* [1]	*Amount*	*Ref.*
Free phenolic hydroxyl	~20% higher	25-35/ 100 C9	12, 13
Methoxyl groups	~20% lower	Variable:	1
Catechol	Formed in pulping	3/100 C9	14
Aliphatic hydroxyls	~60% lower	40/100 C9 [2]	4, 15
Aliphatic carbonyls	Destroyed in pulping	Negligible	3, 16
Aliphatic carboxyls	Formed in pulping	5/100 C9 [2]	4, 5
Aliphatic reduced units	Higher	Variable	3

1 Approximate differences for a residual kraft lignin from a 30 kappa pulp

2. Converted literature values to groups/100 C9 using 185g/mole as C9 unit

Numerous studies with individual model compounds have demonstrated that only those structures containing a free phenolic hydroxyl group react to any extent under oxygen bleaching conditions. The reason for this slow reactivity is that the initial step in ring cleavage reactions by oxygen is the metal catalyzed formation of phenoxy radicals from phenoxy anions (*17*). Because of the lack of reactivity by non-phenolic model compounds, the majority of studies on ring cleavage by oxygen have focused on compounds with free phenolic hydroxyl groups. However, a non-phenolic model compound has been degraded by oxygen in the presence of a compound containing a free phenolic hydroxyl group and excess iron (*18*). As will be discussed later, this increase in reactivity of the non-phenolic hydroxyl model compound was caused by the generation of oxygen

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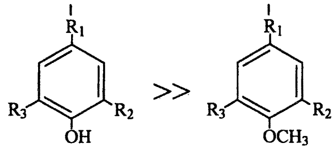
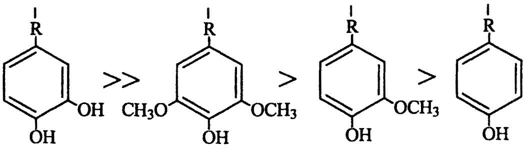
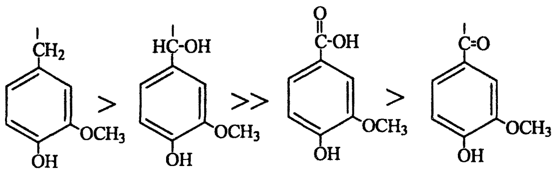
radical species in the reaction of oxygen with the free phenolic model compound.

Methoxyl/Catechol

The number of methoxyl groups per phenylpropane unit in residual lignin is a function of the raw material but is also affected by alkaline demethylation reactions that form catechol groups during pulping (*19*). The number of catechol groups in residual lignin has been determined through permanganate oxidation (*14*) and ^{31}P NMR (*5*) to be approximately 3/100 phenylpropane units. Several labs (*20*, *21*, *22*) have studied the reactivities of model compounds of the catechol, syringyl, guaiacyl, and p-hydroxy types under alkaline oxygen bleaching conditions. Catechol containing compounds are by far the most reactive. The amount of catechol groups present in residual lignin following oxygen bleaching, however, is not significantly changed indicating that these groups are also formed during oxidation (*5*). Ring cleavage in syringyl model compounds was found to be approximately ten times faster than guaiacyl compounds which were ten times faster that p-hydroxy compounds.

Table II. Relative Reactivity of Lignin Model Compounds with Oxygen

Functional Group	*Relative Reactivity to Oxygen*	*Ref*
Phenolic hydroxyl		20
Methoxyl	 R OH OH >> R CH3O OCH3 OH > R OCH3 OH > R OH	20 21 22
Side chain	 CH2 OCH3 OH > HC-OH OCH3 OH >> O C-OH OCH3 OH > C=O OCH3 OH	20 21 22

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Side Chain Substituents

Native lignin contains roughly 100 aliphatic hydroxyl groups per 100 phenylpropane units (*23*). These primary and secondary hydroxyls undergo a variety of reactions during a kraft cook. For example, a large number of the γ carbons containing hydroxyls are lost as formaldehyde resulting in the formation of stilbene and vinyl ether structures. The aliphatic hydroxyl content of dissolved lignin is decreased by approximately 40% (*24*). The reported content (*4*, *15*) of aliphatic hydroxyls in residual lignin is 2 mmol/g (~37/100 C9), which corresponds to approximately a sixty percent decrease.

Native lignin contains carbonyl groups in α-ketone and coniferaldehyde type structures in amounts of approximately 8/100 phenylpropane units (*25*). Analysis of residual lignin after kraft pulping has shown that these carbonyl groups are nearly nonexistent (*3*, *16*). Carbonyl groups in quinone structures are not included in this number.

Native lignin is essentially free of carboxyl groups. The kraft pulping process introduces roughly 5 groups/100 phenylpropane units into residual lignin (*4*, *5*). The exact nature of these groups is not known. During oxygen bleaching, a significant number of new carboxylic acids groups are introduced through side chain and ring cleavage reactions. Muconic acid structures are the primary ring cleavage products but are very reactive under oxygen bleaching conditions and should be present in only very minor amounts (*26*). Side chain cleavage reactions produce phenolic carboxylic acids of the vanillic acid type.

Native lignin contains a limited number of aliphatic saturated CH_2 and CH_3 groups. The amount of these reduced units increases significantly in residual and dissolved lignins (*3*). Information on the reaction mechanisms responsible for the formation of these structures is limited.

Several laboratories have studied the effect of side chain constituents on the reactivity of model compounds to oxygen (*20, 21*, *22*). It has been found that structures containing methylene or methyl groups are quite reactive followed by alcohols, carboxylic acids, aldehydes and ketones. The latter three groups of compounds are only slightly reactive under oxygen bleaching conditions. The reaction rate of these compounds can be shown to be a function of the electron donating ability of the side chain constituents.

Dimeric Model Compound Reactivity: Effect of Linkages

Dimeric model compounds have been studied to investigate the susceptibility of lignin linkages to oxidation. Unfortunately, the exact nature of these linkages in kraft residual lignin has yet to be fully determined. Therefore, the importance of each linkage to the susceptibility of lignin to degradation by

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oxygen is not clear. Information on the current knowledge of the frequency of residual lignin linkages is presented in Table III. The reactivity of model compounds containing these linkages under oxygen bleaching conditions is presented in Table IV.

Uncondensed Units

The β-O-4 linkage is by far the most abundant linkage in native lignin. Significant cleavage of this linkage occurs during the kraft pulping process (*27, 28*). Analysis of residual lignin using either acidolysis (*2*) or ^{13}C NMR (*4*), have shown that up to 85% of these linkages are cleaved in a pulp cooked to a 30 kappa. Depending upon the sulfidity of the cook, a certain number of these types of units are converted to vinyl ethers through the elimination of formaldehyde (*29*). Estimates of the amount of these groups in residual pulp is 0.5-1.0% of the total linkages (*2*).

Condensed Units

The number of β-5 linkages in native lignin has been determined through ^{1}H NMR (*30*) and through a combination of thioacidolysis and ^{31}P NMR (*31*) to total approximately 7-8% of the linkages. This is slightly lower than the value of 9-12% obtained using permanganate oxidation (*32*). Analysis of pulp samples using this latter system, has shown that the relative number of β-5 linkages in residual increases slightly through the cook (*14, 33*).

The amount of β-1 linkages in native lignin has recently been quantified by ^{1}H NMR at 2-3.5% of the total linkages (*34, 35*). This is significantly lower than the 7% previously determined through acidolysis (*32*). The amount of these linkages in residual lignin has not been reported although a certain percentage will be lost through conversion to stilbene structures (*36*). The amount of stilbenes in residual lignin has been estimated using an UV analysis to be approximately 3% of the total linkages (*11*).

The percentage of 5-5 linkages in native softwood lignin has been determined through a variety of different methods. A range of 9.5-11% of the total linkages was generated through permanganate oxidation studies (*32*). Using a combination of thioacidolysis and ^{31}P NMR (*31*), it was determined that 16% of the phenylpropane units in softwood MWL were linked 5-5. A higher value of 24-26% of the phenylpropane units was generated through the use of ^{13}C NMR (*37*). It has been determined that a large portion of these linkages is found in dibenzodioxocin structures (*38*). Analysis of kraft residual lignin samples using permanganate oxidation has shown that the percentage of 5-5

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linkages is somewhat increased over native lignin (*14*, *33*). This is also true for 4-O-5 linkages that make up approximately 4-5% of the total linkages in MWL (*31*, *39*).

The number of condensed structures of the diphenylmethane type formed during alkaline cooking is a matter of debate. Analysis of residual lignin samples using various methods have generated values from roughly 5% to as high as 60% of the total linkages (*39*, *40*). The validity of the method used in the determination of the high values has been questioned (*41*).

Table III Frequency of Linkages in Kraft Residual Lignin

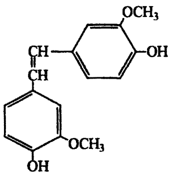
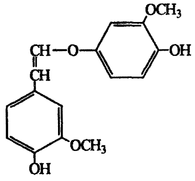
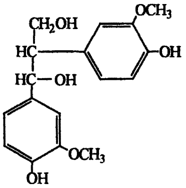
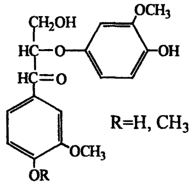
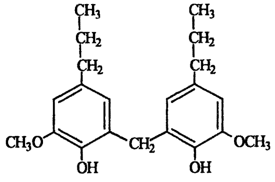
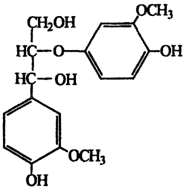
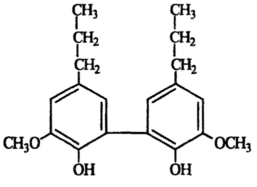
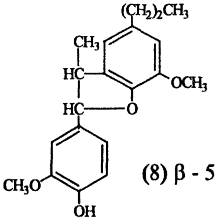
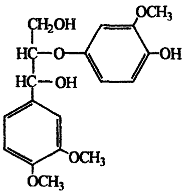
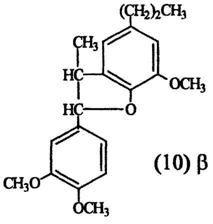
Linkage	*MWL (% of linkages)*	*Ref.*	*Residual Lignin*[1] *(Relative to MWL)*	*Ref.*
β-O-4	48	34	85% lower	2
β-5	7-8	30, 31	Slightly Greater	14, 33
	9-12	32		
β-1	<2	34		
	3.5	35		
5-5	10-11	32		14, 33
	(16)[2]	31	Slightly Greater	
	(24-26)[2]	37		
4-O-5	4-5	31, 39	Slightly Greater	14, 33,
β-β				
Stilbenes	Negligible		~3% of linkages	11
Vinyl Ethers	Negligible		0.5-1% of linkages	2
DPM[3]	Negligible		Debatable Amounts[4]	39, 41,40

1. 1 Approximate differences for a residual kraft lignin from a 30 kappa pulp
2. Values represent % of phenyl propane units containing 5-5 linkage.
3. Diphenylmethane structures of various linkages
4. Amounts ranging from ~5% to greater than 60% have been reported

Susceptibility of Dimers to Oxidation

Several laboratories have studied the susceptibility of dimeric compounds to degradation by oxygen. Unfortunately, different conditions have been used in each study, which makes comparison of the relative stability of different structures difficult. This is because the oxidation rate in these reactions is highly affected by system parameters such as pH, temperature, oxygen charge, and reactant charge (*42*). Certain generalities on the relative reactivity of different linkages can be drawn from this data. A series of model compounds are listed in Table IV in relative order of susceptibility to oxidation.

Table IV Relative Susceptibility of Model Compounds to Oxygen

Model Compounds Tested *(Approximate order of reactivity)*	*Ref.*
Very Reactive  (1) Stilbene  (2) Vinyl Ether	43
Somewhat Reactive  (3) β-1  R=H, CH₃ (4) β-O-4, α-carbonyl  (5) DPM  (6) β-O-4, α-hydroxyl  (7) 5 - 5  (8) β - 5	42 44 45 46 47 48
Non - Reactive  (9) β-O-4, α-hydroxyl  (10) β - 5	46 48

The most reactive dimeric compounds are of the stilbene type closely followed by vinyl ether structures. Stilbene structures are rapidly degraded by oxygen under alkaline conditions even at temperatures as low as 45°C (*43*). When subjected to alkaline oxidation, degradation of phenolic stilbenes occurs across the double bond forming phenolic aldehydes (*49*). Vinyl ether structures

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also cleave across the double bond. Although still quite reactive, vinyl ethers oxidize over one hundred times slower than stilbenes.

The model compounds in the second row of Table IV are all significantly less reactive with oxygen under bleaching conditions than the stilbenes and vinyl ethers. Although these compounds react an order of magnitude slower than the vinyl ether, amongst this group there is not a significant difference in reactivity. The β-1, β-O-4 (α-carbonyl), and the diphenylmethane-linked models react roughly twice as fast as the β-O-4 (α-hydroxyl), 5-5, and the β-5 linked compounds (*42*, *44*, *45*, *46*, *47*, *48*). The reactivity of different linkages in diphenylmethane structures has been shown to decrease in the order α-5, 5-5, α-6 and α-1 (*50*). Phenolic aldehydes, alcohols, ketones, and carboxylic acids along with aliphatic acids are the main degradation products.

The etherifed model compound dimers tested were generally unreactive under oxygen bleaching conditions (*46*, *48*). Only the β-O-4 linked dimer containing a α-carbonyl group was reactive (*46*). It is important to note that these compounds were reacted alone. As note earlier the reactivity of etherified units increases in the presence of phenolic compounds.

Reaction Pathways

Model compounds studies have provided insights into the reactions involved in the oxygen degradation of lignin. In general, mechanisms have been proposed based upon reaction products generated from the degradation of lignin model compounds with molecular oxygen and with the various oxygen radical and anionic species which are generated during oxygen bleaching. Several excellent reviews have been written recently on the oxygen species present in the reaction (*51*, *52*) and on the mechanisms involved in lignin degradation (*53*, *54*, *55*). Therefore, this chapter will not cover these mechanisms in depth but rather provide a general summary of the information.

Table V. Oxygen Species Present in Bleaching

Oxygen Species		*Anionic Form*		*pKa*
Oxygen	O_2			
Hydroperoxy Radical[1]	$HO_2\cdot$	Superoxide Anion Radical	$^{-}O_2\cdot$	4.8
Hydrogen Peroxide	H_2O_2	Hydroperoxy Anion	HO_2^{-}	11.6
Hydroxyl Radical	$HO\cdot$	Oxyl Anion Radical	$^{-}O\cdot$	11.9

1. Negligible amounts present under alkaline bleaching conditions

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Oxygen Species

Under the conditions typically used in oxygen bleaching, there are a large number of oxygen species present (Table V). The reaction of oxygen with a lignin model compound under alkaline conditions generates a superoxide radical anion through a one-electron transfer from the model compound to oxygen. This is generally the rate-determining step of the oxidation and requires the presence of metal ions (*17*) or elevated temperatures (*56*). This superoxide radical can undergo a metal catalyzed dismutation (*57*) forming hydroperoxy anion that can further undergo a metal catalyzed disproportionation forming a hydroxyl radical. A listing of some of the interconversion reactions these oxygen species can undergo is shown in Figure 1. Certain of these reactions are extremely rapid under oxygen bleaching conditions while others require the presence of metals or protons to catalyze the reaction (58)

$$\bullet O_2^- + HO\bullet \longrightarrow HO^- + O_2$$

$$HO_2^- + HO\bullet \longrightarrow HO^- + \bullet O_2^-$$

$$HO\bullet + HO\bullet \longrightarrow H_2O_2$$

$$\bullet O_2^- + \bullet O_2^- + H_2O \longrightarrow HO_2^- + O_2 + HO^-$$

$$\bullet O_2^- + H_2O_2 \longrightarrow HO^- + HO\bullet + O_2$$

$$H_2O_2 + HO_2^- \longrightarrow H_2O + HO\bullet + \bullet O_2^-$$

Figure 1. Reactions of oxygen species under alkaline bleaching conditions

Of the oxygen species listed in Table V, only the hydroxyl radical and its conjugate base are strong oxidants (Table VI). The oxygen molecule, or what is referred to as dioxygen in biological fields, is a weak oxidant. Therefore bleaching is run under alkaline conditions so that ionized phenolic hydroxyl groups on lignin will furnish the high electron density needed to initiate a one electron transfer. Superoxide radical anion acts as a weak oxidant and also as a reducing agent. It is a strong Brønsted base (*59*) in aqueous solutions while hydroperoxy anions and oxyl anion radicals are weak Brønsted bases. Of the oxygen species listed in Table V, only the hydroperoxy anion functions as a nucleophile in bleaching while the other oxygen species function as electrophiles.

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Table VI. Standard reduction potentials for oxygen species in water at pH = 14 (52)

	Standard Reduction Potentials
$O_2 + e^- \rightarrow \cdot O_2^-$	- 0.33 volts
$\cdot O_2^- + e^- \rightarrow HO_2^-$	0.20 volts
$HO_2^- + e^- \rightarrow HO\cdot$	- 0.03 volts
$HO\cdot/\cdot O^- + e^- \rightarrow HO^-$	1.77 volts

Hydroxyl Radical

Although an electron transfer to oxygen is the first step in the oxidation of lignin model compounds, the reactivity of the hydroxyl radical justifies it receiving top billing in any discussion on oxidation. Generation of hydroxyl radicals has been accomplished through chemical methods (Fenton's chemistry), radiolysis of water, UV photolysis of hydrogen peroxide, and vacuum UV photolysis of alkaline solutions containing N_2O (*57*). Through analysis of the products of the reaction of hydroxyl radicals with a large number of monomeric and dimeric lignin model compounds (*60*, *61*, *62*, *63*) a series of reactions pathways has been proposed. The first step in all of these pathways is the formation of a hydroxycyclohexadienyl radical (Figure 2) through the break down of a short lived charge-transfer adduct formed through a rapid addition of the hydroxyl radical to the π-electron system of the aromatic ring (*53*). Ring substituents effect the positioning of the hydroxycyclohexadienyl radical and therefore also the degradation pathways.

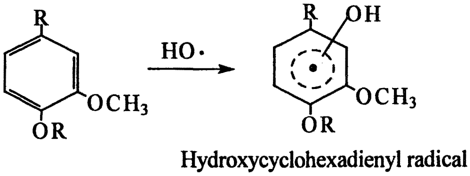

Figure 2. Formation of hydroxycyclohexadienyl radical.

The hydroxycyclohexadienyl radical can lose a hydroxyl anion forming a cation radical which will undergo a variety of different reactions (Figure 3). It is important to note that hydroxyl radicals can react with both phenolic and non-phenolic compounds. Phenolic cation radicals can undergo disproportionation

[1], phenolic coupling [2], and side chain oxidation [3]. Non-phenolic structures will undergo side chain oxidation [3] and Cα-Cβ cleavage [4].

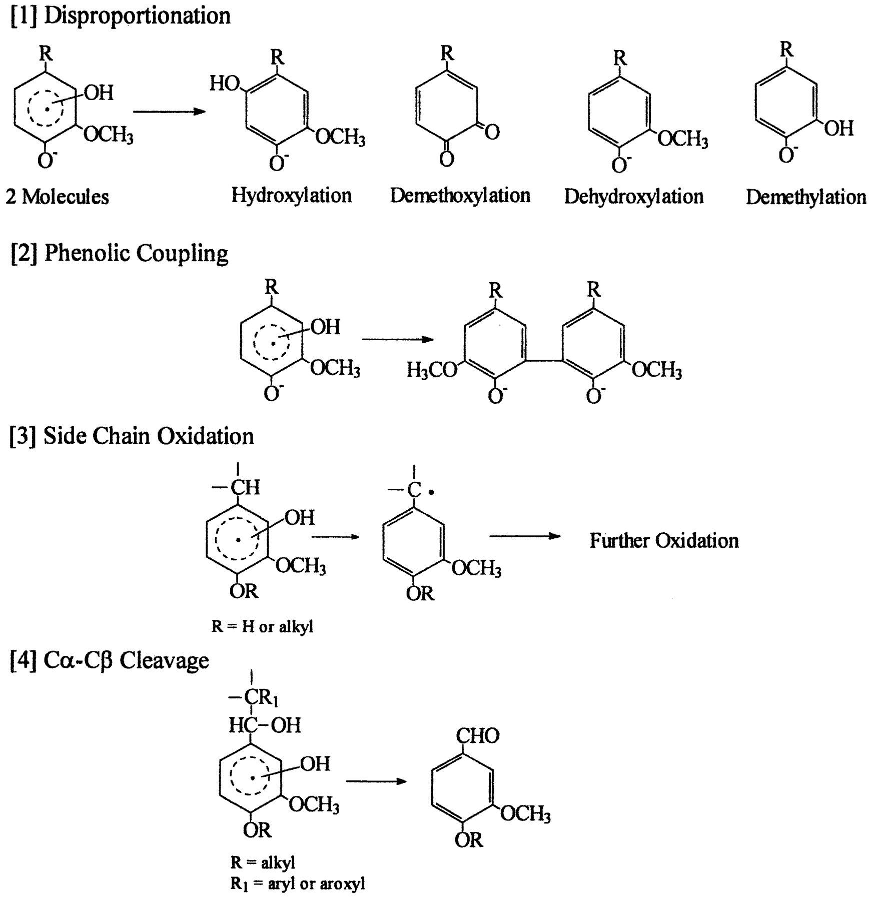
[1] Disproportionation
R
OH
OCH3
O-
HO
O
2 Molecules
Hydroxylation
Demethoxylation
Dehydroxylation
Demethylation
[2] Phenolic Coupling
H3CO
[3] Side Chain Oxidation
-CH
-C•
OR
Further Oxidation
R = H or alkyl
[4] Cα-Cβ Cleavage
-CR1
HC-OH
CHO
R = alkyl
R1 = aryl or aroxyl

Figure 3. Reactions of hydroxycyclohexadienyl radical. (Adapted with permission from reference number 53)

When oxygen is present, the hydroxycyclohexadienyl radical can lose an electron to oxygen forming a superoxide radical. Following the loss of a proton and rearrangement, the model compound will either become hydroxylated [5], demethoxylated [6], or will suffer side chain cleavage [7] in the case of models containing an olefinic structure (Figure 4). Oxyl anion radical will not react with aromatic structures/ hydroxyl reaction with phenolic and non-phenolic structures.

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The rate of degradation of lignin model compounds with hydroxyl radical is much slower at a pH above the pKa (11.9) of the hydroxyl radical (*53*). It has been determined that the oxyl radical anion will cause side chain cleavage reactions but will not react with aromatic or olefinic structures.

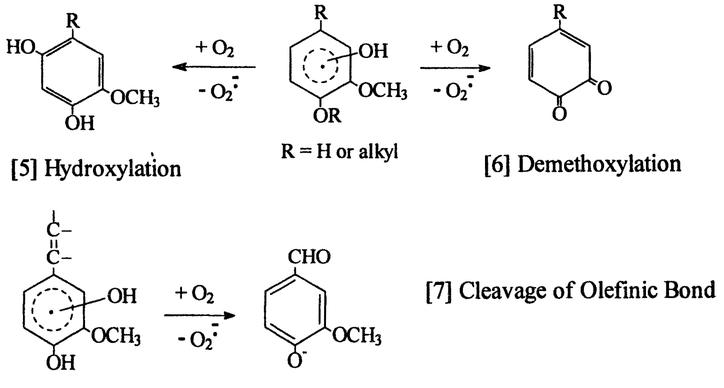

Figure 4. Reactions of hydroxycyclohexadienyl radical with oxygen. (Adapted with permission from reference number 53)

Superoxide Radical Anion

Early studies on the reaction of superoxide radical anion with lignin model compounds were undertaken with potassium superoxide in non-aqueous solutions (*64*). In order to study the reactions under aqueous conditions, radiolysis of an alkaline solution of H_2O_2 was used (*65*) which also generated hydroxyl radicals. As stated previously, the superoxide radical anion is a weak oxidant and as such does generally not degrade lignin directly. Rather, the superoxide radical (Figure 5) will combine with substrate radicals forming peroxide anions that will either cleave the aromatic ring forming muconic acids [8], form oxirane structures [9], or result in the cleavage of olefinic structures [10]. Exceptions to this are catechol structures that are directly degraded by superoxide radical (*64*).

Hydroperoxy Anion

The reactions of hydrogen peroxide/hydroperoxy anion with lignin compounds have been extensively studied and many reviews written (*66*, *67*, *68*). As a review of the chemistry involved in this process is presented as a

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separate chapter in this publication, only a cursory mention of the chemistry will be presented here. Under alkaline conditions, hydrogen peroxide/hydroperoxy anion will react as nucleophiles with carbonyl and conjugated carbonyl structures forming a variety of carboxyl containing degradation products (*69, 70*).

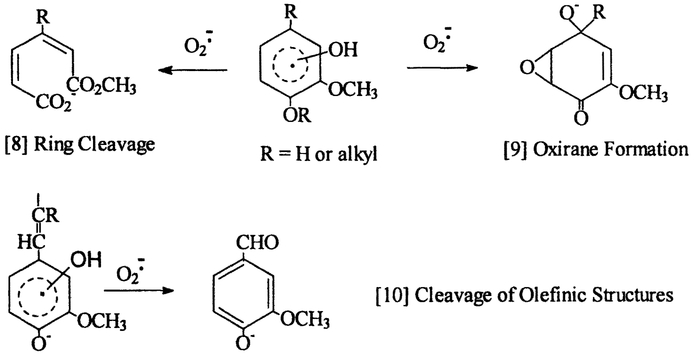

Figure 5. Reactions of superoxide radical anion with superoxide radical anion. (Adapted with permission from reference number 55).

Model Compounds versus Lignin Oxidation

It is important when reviewing the chemistries of the oxygen degradation of lignin model compounds, to compare these results to the actual degradation of lignin in pulp. The structure of residual lignin in pulp after oxygen bleaching has been studied by several laboratories (*5, 6, 71, 72,*). It can be concluded from a comparison of model compound and lignin studies that most of the model compound results are valid when describing lignin degradation. For example, condensed structures containing 5-5 linkages, which are oxidized slowly in model compounds, are shown to increase in frequency in residual lignin after pulp bleaching (*73*). Demethoxylation, ring cleavage and a reduction in phenolic hydroxyl groups are also shown to occur in both model compound and lignin studies. What is different, however, is that the rate of degradation in actual lignin systems is much slower and the extent of degradation much lower. Therefore, more research is needed to truly understand lignin oxidation.

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Chapter 3

Chemistry of TCF-Bleaching with Oxygen and Hydrogen Peroxide

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A variety of analytical methods, applied on pulp and isolated lignin samples, has been used to obtain information about the chemical changes that take place in an oxygen and a hydrogen peroxide stage respectively. Modern 2-D NMR techniques, in particular, have proven to be of particular importance. The major portion of an oxygen delignification stage is a reinforced extraction of a lignin which resembles structurally the lignin being dissolved in the last part of the cook. In hydrogen peroxide bleaching, a limited oxidation of chromophoric structures is accompanied by a delignification reaction starting from intact phenolic phenylpropane units of the benzyl alcohol type. The degree of delignification and bleaching in a P-stage seems to be dependant on the amount of remaining β-O-4 structures in lignin after the cook.

The kraft pulping process is used worldwide for the production of a variety of unbleached and bleached pulps from both softwood and hardwood. Despite the fact that major efforts have been put into further development of the process, bleached kraft pulps still require large quantities of bleaching chemicals. It is

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

also difficult to reach the highest brightness values at least for softwood pulps unless chlorine containing bleaching chemicals are used. From a system closure point of view, it would be of advantage if bleached pulps could be produced using only oxygen based bleaching chemicals. At present, such bleaching sequencies are not cost effective, however.

Recently, the bleachability of kraft pulps has been addressed by several research groups (*1-3*). So far, no firm conlusions about chemical factors affecting this important issue can be made, however, although a correlation between the content of β-O-4 structures in the residual lignin after the cook and the bleachability in an OQP-type of sequence has been identified (*1, 4*). Obviously, more information is needed about the structure of residual lignin in unbleached as well as in bleached kraft pulps of different types.

In the present work, lignin has been isolated from unbleached and oxygen bleached kraft pulps respectively and the lignins subjected to various analytical methods such as ^{13}C and 2-D NMR, oxidative degradation and phenol determination. The results obtained are added into a general description of the chemical features of residual lignin thought to play an important role in the bleaching of kraft pulp with oxygen and hydrogen peroxide respectively.

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Experimental

Pulp Samples

The pulp samples used in this work are from Scandinavian softwood, predominantly spruce, and prepared either by laboratory cooking using a sulfidity of 35% and 18% effective alkali at 170 °C or obtained directly as mill samples.

Oxygen delignification has been done in the laboratory at 100 °C and with variation of oxygen pressure and charge of alkali in order to reach the desired kappa numbers (see Table II). One mill sample has also been used.

Isolation of Lignin

Isolation of the residual lignin from various pulps has been done as described previously (*5*)

Oxidative Degradation with Permanganate

Oxidative degradation with permanganate was done as described in (*6*) directly on unbleached laboratory made pulp with a kappa number of 30, on the corresponding isolated residual lignin, on an oxygen delignified kraft pulp with a kappa number of 18 and finally on the corresponding isolated residual lignin.

Analysis of Phenols

Analysis of the content of phenolic hydroxyl groups was done directly on pulp samples and on the corresponding isolated dissolved lignin using the procedure described in (*7*). All data are taken from Refs. *8* and *9*.

NMR Analyses

The 2-D HSQC sequence was applied on all lignin samples and run such that quantitative information about some of the various structural units could be obtained. Details of the method will be published elsewhere (*10*).

Results and Discussion

The Kraft Pulp Lignin

The residual lignin in kraft pulps has undergone several chemical changes as the result of exposure to high temperature, high alkalinity and the presence of hydrosulfide ions. In addition, the pulping system contains a variety of both solid and dissolved structures/fragments from lignin and polysaccharides which may interfere with the desired lignin fragmentation reactions. Some of the features of the residual lignin are summarized in Scheme 1.

- **A low remaining amount of β-O-4 structures (*11*)**
- **Linkages between lignin and polysaccharides (*12, 13*)**
- **The presence of reduced structures such as CH_2 and CH_3 (*14, 15*)**
- **A high degree of discoloration (*16, 17*)**
- **A successive increase of "condensed" structures with the degree of delignification (*18*)**
- **An uneven distribution of lignin across the fiber wall (*19*)**

Scheme 1. Some characteristics of residual lignin in kraft pulps.

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The most important lignin reaction during kraft pulping, the cleavage of β-O-4 structures, takes place in both phenolic and non-phenolic units albeit with greatly different rates of reaction (*20*). Towards the end of the cook, there is, however, still a measurable amount of intact β-O-4 structures in the residual lignin (*11*). In the corresponding dissolved lignin, such structural units are also present but at a somewhat lower amount. In previous work (*4*), it was shown that the pulping conditions, i.e. the concentration of hydroxyl and hydrosulfide ions, temperature and ionic strength, could influence the level of remaining β-O-4 structures to a considerable extent. A low H-factor to reach a given kappa number was found to result in a high remaining amount and this, in turn, affected the bleachability in OQP-sequencies positively.

The isolation of residual lignin from kraft pulps using enzymatic hydrolysis of polysaccharides always results in a lignin still containing some 6% sugars. In early work in this area (*12*), it was suggested that alkali stable ether linkages between e.g. benzylic carbons in the lignin and the terminal hydroxymethyl group in sugars exist. Later, these types of lignin samples have been subjected to a variety of chromatographic and analytical methods (*13*) resulting in the conclusion that lignin-carbohydrate linkages are present in kraft pulps and that all the wood polysaccharides seem to be involved although xylan is predominant.

After a kraft cook, the resulting liquor contains a large variety of low molecular weight lignin derived compounds (*21, 22*). Some of these contain reduced side chain groups, i.e. methylene and methyl groups. It has been suggested that such groups are formed through reduction of intermediate quinone methides by diarylmethane (*16*) or reducing sugar units (*23*) although the experimental evidence are still scarce. Analysis of isolated dissolved (*14*) as well as of residual lignin (*15*) from kraft pulping using the ^{13}C DEPT NMR sequence reveals that the polymeric lignin also contains the corresponding reduced side chain groups. The presence of such groups can be assumed to further decrease the reactivity of the lignin in both hydrolytic and oxidative processes.

So far, our attempts to identify the reduced side chain structures in the polymeric lignin in more detail by using NMR have been unsuccessful. In earlier work it was suggested that condensation reactions between formaldehyde, liberated from terminal hydroxymethyl groups, and reactive phenolic structures resulting in the formation of diarylmethane structures could be one source of the reduced structures (*14, 24*). By applying the HSQC NMR sequence on isolated residual lignin as well as on the model compound di-(2-hydroxy-3-methoxy-5-methyl)-phenylmethane, it could be shown, however, that such methylene groups do not exist in the residual lignin. This type of methylene group gives the values 28.9 and 3.82 ppm respectively for the carbon and proton shifts; a shift range that is completely empty in the corresponding analysis of the residual lignin.

In technical operations, the target kappa number of the unbleached pulp can be reached in several different ways with pulp quality, e.g. strength, always being the determining factor. Thus, the optimal conditions for obtaining a high bleachability may not always be compatible with the conditions for high quality. Therefore, it is necessary to investigate the structure of the residual lignin in pulp samples which have been prepared under very well controlled conditions so as to minimize the influence of unsystematic changes. In our work, it has been found that the remaining amount of β-O-4 structures (*4*) and the color of the unbleached pulp (*25*) both exert an influence on the bleachability of the pulp.

With few exceptions, work aiming at elucidating structural features of residual lignin requires an isolation procedure prior to analysis. At present, two alternatives are available, viz. acid hydrolysis of the pulp with 0.1 M HCl for 2 h (*5*) and enzymatic hydrolysis with cellulolytic enzymes for 48 h (*13*). The former method results in a lignin which contains virtually no carbohydrates, has a somewhat increased amount of phenolic hydroxyl groups and a yield of around 50-60%. The enzyme lignin, on the other hand, can be obtained in high yield (80% from unbleached pulp) but it contains around 6% carbohydrates and varying amounts of impurities from proteins. In our work, the acid hydrolysis method has been used.

In order to verify that the isolation of lignin by acid hydrolysis does not induce condensation reactions between C-6 and C-α in an adjacent lignin unit, unbleached kraft pulp, the corresponding isolated lignin, the same pulp after an O-stage and the isolated lignin were all subjected to analysis by oxidative degradation. As shown in Table I, the relative distribution of the individual degradation acids was in good agreement between the pulp and the corresponding isolated lignin sample although the total yield of acids was higher for the lignin samples. No indication of an increased yield of metahemipinic acid, the expected product of condensation reactions, could be seen. The relative distribution of degradation acids shown in Table I, further indicates that the residual lignin after the oxygen stage contains a slightly higher relative amount of condensed lignin structures of the 5-5 type (cf *26*). A similar slight tendency can be seen by comparing the lignin going into solution late in the kraft cook. Thus, the lignins obtained from a flow-through reactor at the end of the bulk phase (85-93%) and from the residual delignification phase (93-95%) show similar differencies (*18*).

It is well-known that the concentration of lignin in the middle lamella in wood fibers is much higher than in the secondary wall. After a kraft cook, these differencies are still present demonstrating the existence of a lignin gradient

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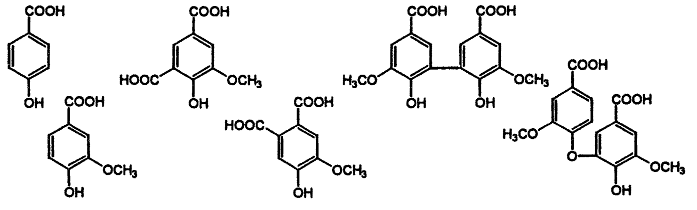
through the fiber wall. This has been shown by subjecting kraft pulp fibers to reaction with chlorine and measuring the relative content of chlorine in the various cell wall layers (*19*).

Table I. Relative frequencies of degradation acids obtained from oxidative degradation of various pulps and lignins (number per 100 aromatic units).



Kraft pulp	2.6	42.3	16.1	6.0	20.0	12.1
Residual lignin	1.4	40.4	16.7	6.5	22.2	12.0
O-delign. kraft pulp	1.2	38.4	18.8	6.0	24.3	11.4
Residual lignin	1.2	33.5	19.1	5.9	26.5	13.3
Diss. lignin, 85-93%[1]	0.7	44.8	19.5	4.2	19.9	10.4
" , 93-95%[1]	0.6	40.0	20.5	4.5	22.0	11.9

[1] Ref. 18

The quantification of β-O-4 structures containing a hydroxyl group in the α-position or being part of the dibenzodioxocin ring system as well as of some other lignin sub-structures such as β-5 and β–β can be done by 2-D NMR using the HSQC-sequence (*10*). For other types of β-O-4 structures e.g. those having an ether function in the α-position the lack of knowledge about exact shift values and the fact that several different types of structures overlap in the NMR spectrum prevent a detailed quantitative analysis. Therefore, the values obtained by this NMR method will give lower numbers for the β-O-4 structures as compared to the corresponding values found in the literature. For Milled Wood Lignin (MWL), the sum of β-O-4 and dibenzodioxocin structures was found to be 39 per 100 phenylpropane units which can be compared to the value 48 given in Ref *27*. Based on NMR data, the difference, 9 units, should constitute different types of α-ether-β-O-4 structures. For β-5 and β–β structures, the

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values obtained by the NMR method are quite close to those found by other methods, i.e. 11 and 2 respectively (cf. *27*) (Table II).

Table II. Number of sub-structures in some isolated lignin samples.

Lignin sample	β-O-4	β-5	β-β
MWL	39[1)]	11	2
Residual lignin, kappa = 30	9-10	5	2
" , kappa = 18	5-7	3	1
Dissolved kraft lignin	5	2	2
Residual lignin, kappa = 26	11	6	2
Residual lignin after an O-stage, kappa = 9.3	18	8	2
Residual lignin after O, 60 min	19	6	2
" , 120 min	16	8	2
" , 240 min	21	8	2
" , 30+60 min	21	11	1

1)Includes α-hydroxy-β-O-4 and dibenzodioxocin structures

As delignification proceeds in the kraft cook, a successive cleavage of β-O-4 structures takes place. Analysis of these by the acidolysis (or thioacidolysis) method gives values in the residual lignin around 15% of the original in wood at a kappa number around 30 (*11*). A corresponing analysis by the HSQC-sequence yields, however, much higher values and at a kappa number of 30, approximately 26% of the α-hydroxy-β-O-4 structures are still present in the (isolated) residual lignin (Table II). Likewise, the isolated residual lignin from a commercial pulp with a kappa number of 26 was found to contain 11 α-hydroxy-β-O-4 structures per 100 phenylpropane units. The higher values obtained with the NMR method can be explained by the fact that the acidolysis method only includes those β-O-4 structures which in the analysis gives rise to a phenolic structure without any linkages in the C-5 and C-6 positions.

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Dibenzodioxocin structures are present in MWL (*28*) at an amount of around 5 per 100 phenylpropane units (*10*). During kraft pulping these are completely degraded and at the end of the cook no remaining such structures can be found by NMR. Among other structures present in lignin, the β–β structures are quite stable and the amount, around 2 per 100 phenylpropane units, seems to be rather unaffected by the cook. This is, however, not the case with the β-5 structures and analysis of the isolated residual lignin gives values around 50% of the original (Table II). Obviously, the well-known conversion of such structures into the corresponding stilbene occurs to a considerable extent (*29*).

The Oxygen Stage

Oxygen delignification is since long a well established method for the further treatment of kraft pulps before bleaching. Since oxygen is readily available, cheap and an environmentally friendly chemical, its expanded use would be highly desirable. The major drawback is the limited selectivity although development in the process technology has resulted in a considerable improvement. Today, degrees of delignification around 65% can be achieved for softwood pulps without any deterioration of quality characteristics.

The lignin reactions in the oxygen stage have been investigated in detail by several research groups. The two most important reactions are the oxidative ring opening of phenolic lignin structures and the formation of radical species from generated hydrogen peroxide. The latter reaction results in a successive depolymerisation of the cellulose and its extent must be minimized. It can be assumed that the direct reaction between a phenolate anion and oxygen is the predominant reaction in the mixer where a good contact between oxygen and pulp can be achieved albeit during a very short period of time (a few sec). During the remaining reaction time in the oxygen stage (around 60 min), hydrogen peroxide may well constitute the major oxidant while oxygen plays a minor role due to the fact that the solubility of oxygen in water is very low. In this part of the stage, the alkalinity of the bleaching liquor corresponds to a pH around 11-12 and hydrolytic reactions can add to the overall degree of delignification.

Quantitative analysis of the isolated residual lignin from oxygen delignified kraft pulps by the HSQC-sequence reveals that as the oxygen stage is prolonged, an increase in the number of β-O-4 structures can be observed (Table II). Among other lignin structures, a slight increase in the β-5 and an unchanged amount of β–β structures were encountered. These observations demonstrate that the lignin present in the unbleached kraft pulp is highly heterogeneous and lignin structures more representative of the dissolved kraft lignin is present at the same time as lignin with a rather "native" structure. This broad gradient of lignin structures is assumed to be partially dissolved but trapped inside the fiber wall, partially still attached as a more or less "true" residual lignin. In the oxygen stage, a preferential oxidation of the lignin fraction having the highest content of phenolic groups and the highest accessibility can be assumed to take place, i.e. the fraction mostly resembling the dissolved lignin.

A further support for this view can be found by analysis of the content of phenolic hydroxyl groups in the residual and dissolved lignins. Thus, the residual lignin in unbleached kraft pulp has a content of phenolic hydroxyl groups around 30 per 100 phenylpropane units (*8, 30*). After the oxygen stage the corresponding residual lignin contains around 10 phenolic units in accordance with the expected behaviour, i.e. a preferential consumption of phenolic structures. The dissolved lignin from the oxygen stage, on the other hand, has a content of phenols of around 40, i.e. a value far above the average value in the unbleached pulp lignin (*8*). Again, the results show that a residual lignin fraction having a high reactivity towards oxygen/alkali is eliminated leaving a far less phenolic and thus more unreactive residual lignin after the oxygen stage.

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Bleaching with Hydrogen Peroxide

Since long, alkaline hydrogen peroxide has been used as an efficient bleaching agent for mechanical pulps. In this process, it is of importance to maintain a high pulp yield and the bleaching is done in such a way that only the brightening effect of hydrogen peroxide is utilized, i.e. an elimination of chromophoric structures without much loss of substance. In kraft pulp bleaching with hydrogen peroxide on the other hand, it is necessary to delignify the pulp since not all chromophores present can be eliminated (*31, 32*). Thus, in addition to the lignin retaining bleaching action of alkaline hydrogen peroxide, a lignin degrading reaction must be utilized. This is done by operating the stage at a high temperature and using a long reaction time. It is also necessary to pretreat the pulp with a chelating agent prior to the peroxide bleaching to remove as efficiently as possible all transition metal ions (*31*). In addition, the presence of magnesium ions is beneficial in order to obtain an optimal stability of the peroxide and thus a high bleaching response (*33*). Addition of silicate, used for stabilizing the peroxide in mechanical pulp bleaching, is less useful, however, since it will prevent the bleaching liquor from being included in the chemical recovery system of the mill.

Recently, it was shown that the lignin degrading reaction could be due to a side chain oxidation of intact phenolic lignin structures able to form a quinone methide structure (*34, 35*), i.e. the same type of chemistry as encountered in alkaline pulping. In the case of peroxide oxidation, the intermediate organic hydroperoxy structure is formed by a reversible addition of a hydroperoxy anion to the quinone methide. Thus, the kinetics for this reaction should have a resemblance with other types of lignin reactions involving quinone methides and, consequently, tempertures around 100 °C or higher are necessary in order to get a reasonable rate of reaction (*20*).

In accordance with the view that peroxide bleaching involves a side chain reaction as the predominant lignin degrading reaction it was recently demonstrated that the residual lignin from OQP-bleached kraft pulp has a methoxyl content which has not changed much as compared to the same analysis after the O-stage (*9*).

The importance of remaining β-O-4 structures in the residual lignin for an efficient bleaching with hydrogen peroxide has been demonstrated by preparing a series of unbleached kraft pulps having approximately the same kappa number but differing in their content of β-O-4 structures. When subjecting these pulps to a simple bleaching sequence involving oxygen and hydrogen peroxide respectively and using an interjacent chelating stage (OQP sequence), it could be shown that a high content of β-O-4 structures in the residual lignin resulted in a much better bleachability of the pulp (*1, 4*). This result is in complete harmony with the results of lignin analysis and the model experiment described above.

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Conclusions

The results from this work indicate that a slight increase of condensed lignin structures such as 5-5 and possibly 4-O-5 structures takes place in the residual lignin towards the end of the kraft cook. Condensation reactions between aromatic units and formaldehyde, cannot be detected in residual kraft lignin, within the limits of our technique. However, the lignin, after pulping, contains saturated carbons, i.e. CH_2 and CH_3. The origin of these is still unknown although a reduction of quinone methide structures during the cook appears to be a reasonable explanation.

The cleavage of β-O-4 structures in lignin during pulping is comprehensive. Despite this however, after the cook a large amount of such structures is still present in the residual lignin. The quantity of these determines the bleachability of the pulpin sequencies of the OQP-type. In the oxygen stage, an oxidative extraction of the pulp takes place, predominantly removing lignin which e.g. for reasons of large molecular size has been trapped in the fiber wall. The remaining lignin after the oxygen stage resembles native lignin containing a rather high amount of β-O-4 structures with phenolic end-group content around 10%. A subsequent bleaching stage with hydrogen peroxide can delignify the pulp provided that the lignin still contains phenolic structures of the benzyl alcohol type. This structural feature is crucial and should be the focus of continuing work in this area.

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Chapter 4

Lignin Reactions during Oxygen Delignification of Various Alkaline Pulps

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The bleachability of pulp is known to depend on the cooking method used for its preparation. The goal of this study was to investigate the effects of the cooking method on the reactivity of lignin during subsequent oxygen delignification in order to find factors which correlate with the observed differences in pulp bleachability. The comparison was made at the same kappa number level both before and after oxygen delignification. The structural differences between the isolated lignins were small. The results indicate that lignin-carbohydrate linkages play an important role in hindering delignification.

Oxygen delignification is now widely applied in pulping, because it allows pulping to be extended further with reasonable selectivity. The practically feasible delignification level in oxygen delignification is about 50% (1), although methods have been proposed to achieve even higher levels (2,3,4).

The main reactions of lignin during oxygen delignification are known (5,6,7). The key functional groups in lignin in this respect are phenolic hydroxyl groups. They are consumed during the reaction, although some, probably condensed structures, have been found to be comparatively stable (8). Carboxylic acids and quinones are formed during the reaction (3,9).

In the present investigation, the effect of pulping method on the response of

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the pulp towards oxygen delignification was studied. The main goal was to find factors in the structures of the residual lignin-carbohydrate complexes (RLCC) which correlate with the observed differences in the bleachabilities of the oxygen-delignified pulps. A conventional kraft pulp (CK), a polysulfide/anthraquinone pulp (PSAQ), and a soda/anthraquinone pulp (SoAQ) were prepared from the same lot of pine chips. A two-stage neutral sulfite pulp (Si) was prepared as a reference for the alkaline pulps (10). All four pulps were oxygen delignified to as near as possible the same delignification level. RLCCs were isolated from the O-delignified pulps and the starting pulps and analysed by a number of chemical and spectroscopic methods.

Materials and Methods

The unbleached pulps were oxygen delignified in a Quantum-Mark reactor at 10% consistency and 90°C for 1h with 8 bar O_2 and the addition of 0.5% $MgSO_4$. The amount of alkali was adjusted so that 50% delignification was expected (11). The properties of the starting pulps and the oxygen-delignified pulps are shown in Table I.

Table I. Properties of the Pulps.

	CK	*PSAQ*	*SoAQ*	*Si*
Kappa number	31.6	33.6	31.7	29.0
Brightness, %	25.8	26.4	26.6	71.4
Viscosity, ml/g	1280	1180	930	1170
Total yield, %	47.0	51.2	47.2	53.9
	CK/O	*PSAQ/O*	*SoAQ/O*	*Si/O*
NaOH charge, %	2.52	3.10	2.65	1.85
Final pH	10.5	10.5	10.4	9.1
Brightness, %	31.6	29.5	30.3	51.3
Kappa number	17.2	18.7	16.3	17.9
Viscosity ml/g	1050	1010	830	1240
Yield, %	97.9	97.9	98.0	95.2

Analysis Methods

Kappa number (SCAN-C 1:77), brightness (SCAN-CM 11:25 and SCAN P 3:93) and viscosity (SCAN-CM 15:88) were determined according to the standard procedures. Total lignin content (Klason+soluble) was determined according to a method in the literature (12). Isolation and purification of residual lignin was performed by enzymatic hydrolysis and protease purification (13). Carbohydrate analysis of the pulps and RLCC samples was performed according to the published methods (14). The hexenuronic acid (HexA) contents were determined from the enzyme hydrolysates of the pulps (15). Lignin kappa number was calculated using the reported finding that 10 mmol HexA/kg pulp is equal to 0.86 kappa units (16). Lignin content based on UV-determination from enzyme hydrolysate was performed as described earlier (13). Nitrogen and methoxyl group analyses were performed in Germany at Analytische Laboratorien. Phenolic hydroxyl groups were determined using the modified differential ionization UV method (13). Molar mass distribution was determined by gel permeation chromatography using Superdex 200 gel and 0.5 N NaOH as eluent. The calculation was based on Na-polystyrenesulfonate standards. The changes in the gel material (17) were accounted for by using an internal standard (phenol) and using fresh column material (replacement every six weeks). The lignin samples were extracted with acetone before analysis.

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Results and Discussion

Oxygen Delignification Efficiency

In cooking, the aim was to prepare pulps with the same kappa numbers. In oxygen delignification, the alkali charges used for the different pulps were varied, again to achieve the same degree of delignification. This was done so that the effects of the pulping method on the response towards oxygen delignification would be emphasized. A previous investigation on eucalyptus pulps was performed using constant conditions (18). The results obtained disagreed in some respects with those from the present investigation. The reasons were probably the differences in wood species, in experimental conditions or in the evaluation methods used for delignification, *e.g.* contribution of hexenuronic acid to kappa number.

Kappa number as such cannot be used to compare lignin contents of pulps cooked with different methods because of differences in the contents of hexenuronic acid. In the present work, these contents were determined and their contribution was taken into account (16). The total lignin contents were also determined.

The delignification levels differed according to the method used to calculate the lignin contents (Table II). The generally used correlation factor 0.15 was used for the conversion of lignin kappa number into lignin content. However, this correlation is actually valid only for unbleached alkaline pulps containing "typical" amounts of hexenuronic acid, as the experimental correlation factor has been determined before the separation of kappa number into lignin and hexenuronic acid could be done, as has been discussed earlier (19). Therefore, the lignin contents of the pulps were determined using a third method: UV-absorbance of the enzyme hydrolysate (13). Even in this method, an assumption is made concerning the absorptivity of residual lignin; 20 l/g cm is used for all pulp types. The determined absorptivities of the residual lignins of the alkaline pulps in fact varied between 20.9 and 22 as will be shown later (Table VI). For the Si-pulps with lower absorptivities, the UV-method can be considered to give the content of aromatic lignin. The calculated correlation factors between the lignin kappa number and UV-lignin were 0.14 for the unbleached and 0.15 for the oxygen delignified CK and PSAQ pulps. For the SoAQ pulps the factors were slightly higher, 0.15 and 0.18 respectively, and 0.13 for both Si-pulps. Higher values (0.18 and 0.21) have been published earlier for unbleached and oxygen delignified CK-type pulps (19). The calculation method based on UV-lignin contents indicated lower delignification degrees compared to the lignin kappa method with constant correlation factor, as seen in Table II.

Based on lignin kappa and UV-lignin reductions, almost the same degree of delignification was obtained for the alkaline pulps with alkali charges of 3.10% (PSAQ), 2.65% (SoAQ) and 2.52% (CK) (Table I) indicating poor response of the PSAQ pulp towards oxygen delignification compared to the two other alkaline pulps. The low degree of delignification for the Si pulp was due to the small alkali charge used, leading also to low final pH.

Isolation of Residual Lignin

Residual lignin-carbohydrate complexes (RLCC) were isolated from unbleached and oxygen-delignified pulps using the enzymatic hydrolysis method. The fractions obtained from the various pulps are shown in Figure 1.

The yields of the fractions depend on the pulp type and lignin content. In all alkaline unbleached pulps, the insoluble EH fraction was the main product, contrary to the two-stage sulfite pulp (Si), in which the residual lignin is more soluble, being found mainly in the pH 2.5 fraction (Figure 2). Some more material was found after further acidification to pH 1, but this was mainly protein with only traces of lignin. Residual lignin in oxygen-delignified pulps is more hydrophilic and thus soluble. The corresponding fractions from the O-stage pulps are shown in Figure 3.

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Table II. Lignin Contents and Delignification Levels of the Pulps.

Pulp	*CK*	*PSAQ*	*SoAQ*	*Si*
HexA kappa	2.4	2.1	1.1	0
Lign. kappa	29.2	31.5	30.6	29.0
Lign. kappa *0.15, %	4.4	4.7	4.6	4.4
UV-lignin, % (13)	4.0	4.5	4.6	3.9
Gravimetric lign., %	3.6	4.2	4.2	3.9
Soluble lign.,%	0.1	+	+	1.4
Pulp	CK/O	PSAQ/O	SoAQ/O	Si/O
HexA kappa	2.5	2.2	1.3	0
Lign. kappa	14.7	16.5	15	17.9
Lign. kappa *0.15, %	2.2	2.5	2.3	2.7
UV-lignin, % (13)	2.2	2.5	2.8	2.3
Gravimetric lign., %	2.3	3.1	1.9	1.6
Soluble lign.,%	0.2	0.2	0.1	1.2
Delignification based on lignin kappa *0.15, %	50	48	51	38
Delignification based on UV-lignin, %	43	45	39	40
Delignification based on tot. lignin, %	32	21	52	47

NOTE: + = under detection limit 0.1%

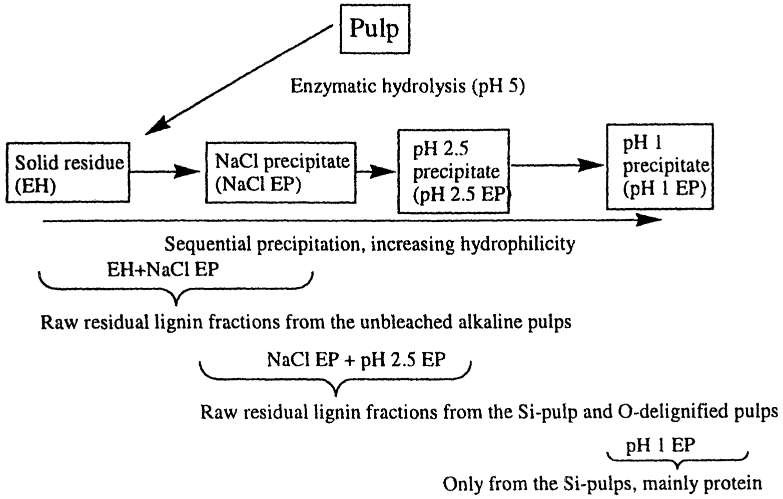
Pulp
Enzymatic hydrolysis (pH 5)
Solid residue (EH)
NaCl precipitate (NaCl EP)
pH 2.5 precipitate (pH 2.5 EP)
pH 1 precipitate (pH 1 EP)
Sequential precipitation, increasing hydrophilicity
EH+NaCl EP
Raw residual lignin fractions from the unbleached alkaline pulps
NaCl EP + pH 2.5 EP
Raw residual lignin fractions from the Si-pulp and O-delignified pulps
pH 1 EP
Only from the Si-pulps, mainly protein

Figure 1. The raw residual lignins obtained by enzymatic hydrolysis.

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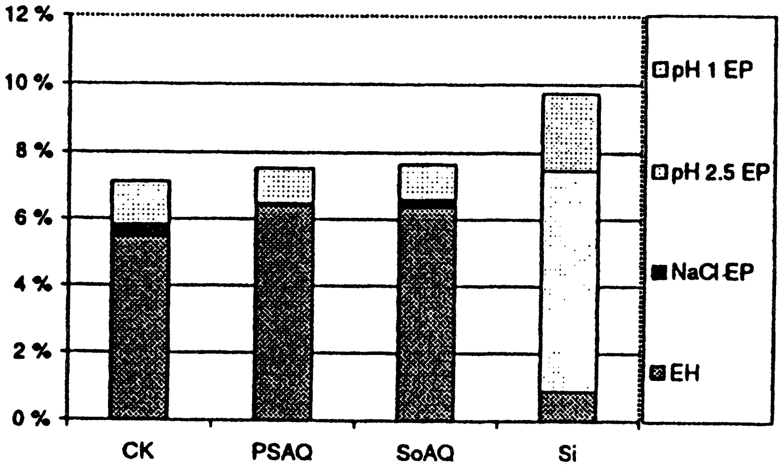

Figure 2. Yields of fractions obtained from unbleached pulps after enzymatic solubilization.

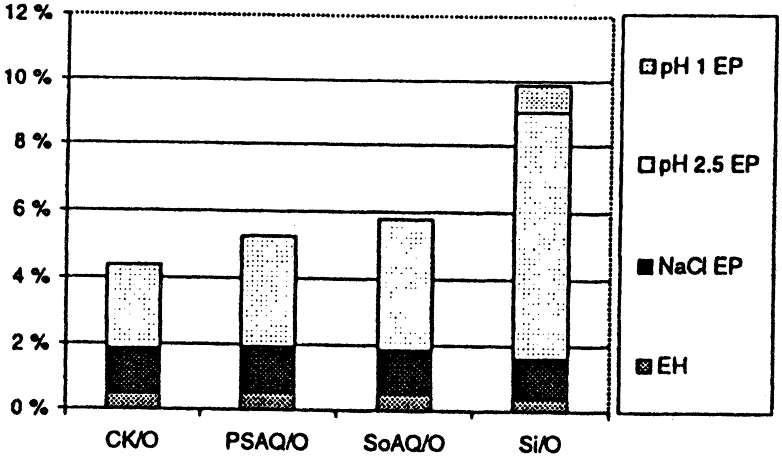

Figure 3. Yields of fractions obtained from O-delignified pulps after enzymatic solubilization

All the fractions were analysed by FTIR, and those which showed the characteristic lignin bands (ca. 1510 cm^{-1}) were purified. The main impurity, especially in the pH 2.5 fractions, was protein from the hydrolysis step. The purification method used was therefore protease treatment with Subtilisine at pH 9.5 according to the scheme in Figure 4.

A modified purification method was used for the Si pulps, because they were too hydrophilic to be precipitated (Figure 5). A similar method has been used earlier for sulfite pulps (20).

The pH 2.5 precipitate of the Si pulp was purified together with the small amount of NaCl fraction. The main lignin fraction after purification was UF>10k. A small amount of insoluble material (UFis) was combined with the main fraction. This combined fraction is referred to as Si RL.

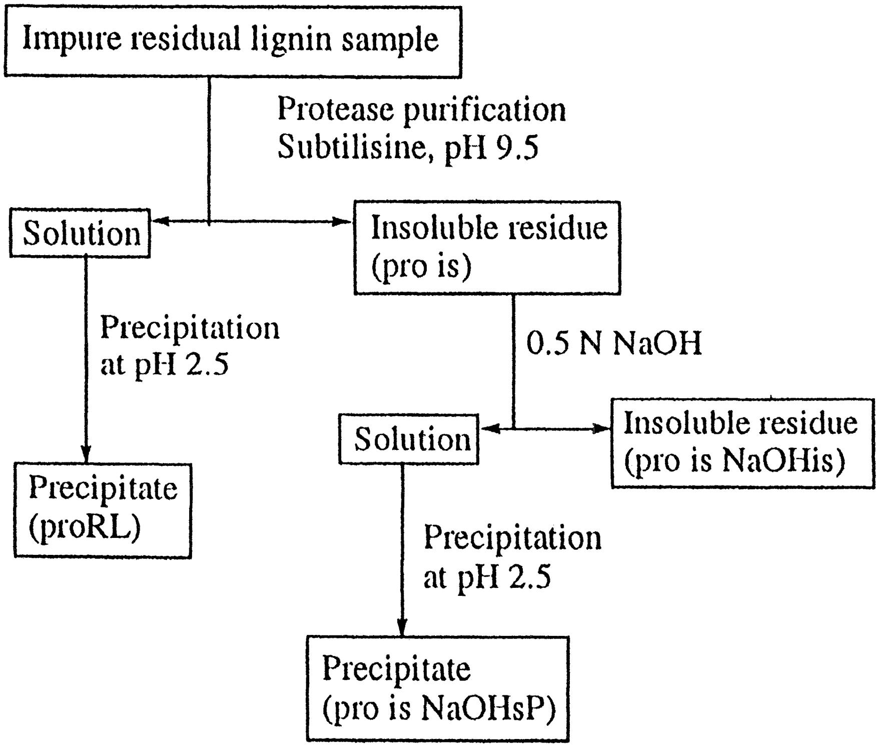
Impure residual lignin sample
Protease purification
Subtilisine, pH 9.5
Solution
Insoluble residue
(pro is)
Precipitation
at pH 2.5
0.5 N NaOH
Precipitate
(proRL)
Solution
Insoluble residue
(pro is NaOHis)
Precipitation
at pH 2.5
Precipitate
(pro is NaOHsP)

Figure 4. Purification of raw residual lignin samples from the alkaline pulps.

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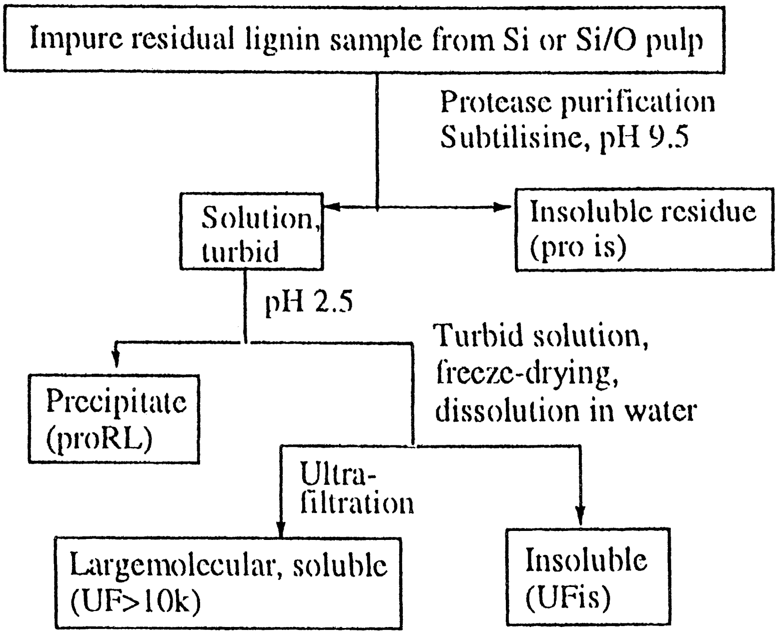
Impure residual lignin sample from Si or Si/O pulp
Protease purification
Subtilisine, pH 9.5
Solution, turbid
Insoluble residue (pro is)
pH 2.5
Precipitate (proRL)
Turbid solution, freeze-drying, dissolution in water
Ultra-filtration
Largemolecular, soluble (UF>10k)
Insoluble (UFis)

Figure 5. Purification of raw residual lignin samples from the Si pulps.

The Si/O pulp yielded a large lignin-rich NaCl precipitate after the hydrolysis. This fraction was very hydrophilic, and after washing it was recovered by ultrafiltration of its water solution (large molecular material 1.3% on pulp, referred to as Si/O NaCl RL). The pH 2.5 precipitate was purified according to the scheme in Figure 5 and lignin was mainly found in the proRL and UFis fractions, which were combined to yield Si/O RL.

The distribution of the purified RLCC fractions of the pulps is shown in Figures 6 and 7. The fraction pro is NaOHis is mainly non-lignin material, and therefore fractions proRL and pro is NaOHsP can be regarded as purified lignin fractions of the alkaline pulps. The analysis results for the pro RL samples are presented here.

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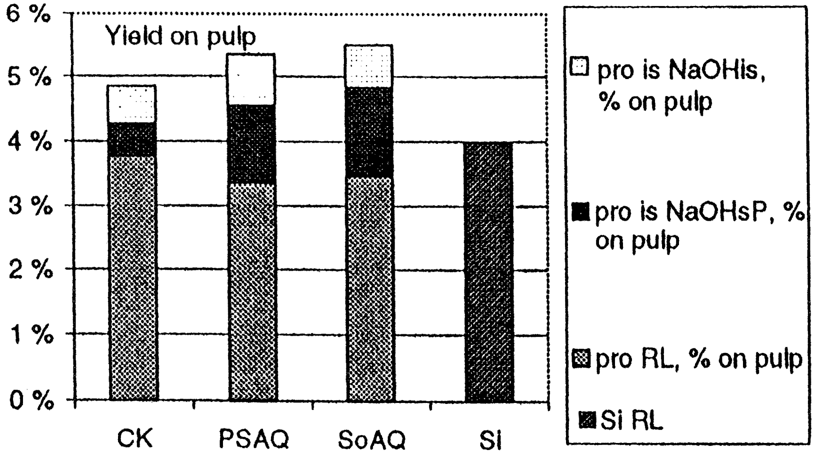

Figure 6. Distribution of the purified RLCC fractions of the unbleached pulps. For explanation of codes see Figure 4 (alkaline pulps) and text above for the Si pulp.

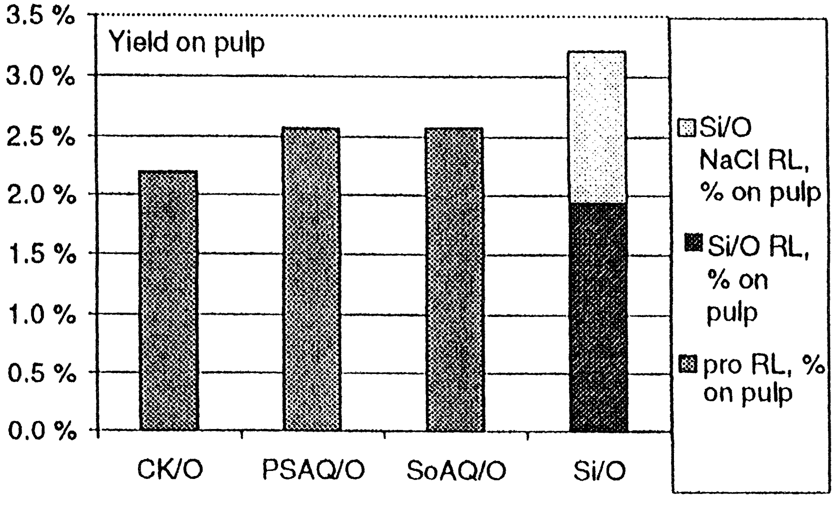

Figure 7. Distribution of the purified RLCC fractions of the oxygen-delignified pulps. For explanation of codes see Figure 4 (alkaline pulps) and text above for the Si/O pulp.

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To evaluate the actual lignin yield of the isolation procedure, the contents of pure lignin in the proRL fractions were calculated by subtracting the determined protein contents (Table VIII) and carbohydrate contents (Figure 10). The same values were used even for the pro is NaOHsP fractions in the case of unbleached pulps, and the yields of the two fractions were summed together. The lignin contents of the pulps shown in Table II were determined as total lignin, calculated based on lignin kappa (constant correlation factor) and on UV-lignin. In Table III, the actual lignin yields in the total RLCCs are compared to the pulp lignin contents. Only the alkaline pulps are evaluated in this respect.

Table III. Lignin Yields Based on Theoretical Lignin Contents

Pulp	*Purified RL, lignin yield (% on total lignin)*	*Purified RL, lignin yield (% based on lignin kappa*0.15)*	*Purified RL, lignin yield (% based on UV-lignin*
CK	102	88	95
PSAQ	94	85	88
SoAQ	100	94	92
CK/O	73	83	82
PSAQ/O	64	86	85
SoAQ/O	106	94	77

The yields based on total lignin are very high in some cases, probably indicating that the actual lignin contents of the pulps are higher than the determined contents. The yields based on lignin kappa number and UV-lignin have less variation between the pulp types.

Analysis of the Lignins

FTIR

The FTIR spectra of the RLCCs of all alkaline pulps were very similar. Lignin was clearly oxidized in the O-stage as judged by the ratio of the intensities of the bands at 1720cm^{-1} (typical for carboxylic acids) and 1510cm^{-1} (aromatic structures). This is demonstrated in Figure 8, which shows as examples the spectra of the RLCCs of the CK and CK/O pulp lignins after subtraction of the protein contaminant (Table VIII). The FTIR spectra of the RLCCs of the O-delignified pulps are shown in Figure 9, again showing the similarity between the alkaline pulps after O-delignification. The contents of carboxyl (incl. non-conjugated carbonyl) groups were also calculated (21). Slight differences in the carboxylic acid contents were found indicating highest contents in the CK lignin and, maybe surprisingly, the SoAQ/O lignin (Table IV).

The spectra of the Si lignins were similar to those presented in literature (22), with the sulfonate groups showing at 1037cm^{-1}. Only a small shoulder in the carboxylic acid region was seen, and it was not quantified. The carboxylic acid region was not intensified even after O-delignification.

Table IV. The Contents of Carboxyl and Non-conjugated Carbonyl Groups in the Lignins by FTIR.

	CK	*PSAQ*	*SoAQ*	*CK/O*	*PSAQ/O*	*SoAQ/O*
CO, mmol/g	0.75	0.37	0.45	1.51	1.10	1.81

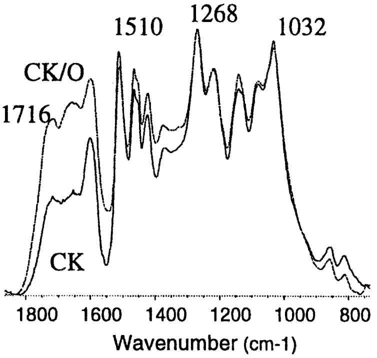

Figure 8. FTIR spectra of the RLCCs of the CK and CK/O pulp RLCCs..

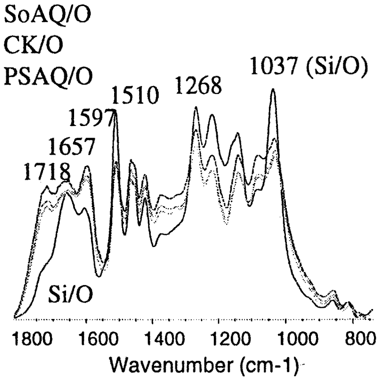

Figure 9. FTIR spectra of the RLCCs of the O-delignified pulps.

Elemental Composition

The elemental composition of the RLCCs was determined. The content of protein impurity (from the cellulolytic enzymes used for the isolation) was calculated from the nitrogen content (N%*6.25). The carbohydrate composition of the samples was determined separately (see below). The proportion of inorganic impurities was estimated as the difference from 100% of the combined weight-percentage of C, H, O, N and S. Only the Si pulps RLCCs contained large amounts of inorganics. The elemental composition of lignin in the samples

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was calculated by subtracting the effect of protein and carbohydrates in relation to their contents. An enzyme blank sample was prepared and analysed for this purpose, and a theoretical composition was used for the carbohydrate part. From the corrected composition, the molecular weight of the C9-unit was calculated, and the methoxyl and phenolic hydroxyl group contents (see below) were calculated both relative to the weight of lignin and relative to the number of C9-units. The corrected elemental compositions of the lignins are shown in Table V. Again, the similarity of the alkaline pulp lignins is evident in both the unbleached and oxygen-delignified pulp series. The main difference is seen in the sulfur content, which naturally is higher in the lignins originating from pulps cooked using sulfur chemicals than in the soda pulp lignins. The lignin remaining in the pulp after the oxygen stage has practically the same sulfur content as before the oxygen stage suggesting the presence of stable C-S linkages in the residual lignins. However, the lignin is more oxidized after this stage. The Si lignins contain large amounts of sulfur due to sulfonation. This is also probably the main reason for the increase in their oxygen contents. The sulfur contents are close to those given in the literature for spruce Si residual lignin (22).

Table V. Elemental Composition of the Residual Lignins.

RLCC	*MW C9*	*C %*	*H %*	*N %*1)	*O %*	*S %*
CK proRL	170.4	63.38	5.98	0.07	29.41	1.12
PSAQ proRL	169.1	63.85	6.18	0.07	28.82	1.04
SoAQ proRL	168.8	63.97	6.11	0.08	29.66	0.18
Si RL	192.4	56.15	6.11	0.58	33.15	3.88
CK/O proRL	175.6	61.49	6.13	0.18	31.28	0.88
PSAQ/O proRL	177.0	61.02	6.13	0.18	31.70	0.94
SoAQ/O proRL	174.7	61.84	5.95	0.20	31.81	-0.20
Si/O NaCl RL	204.4	52.84	5.63	0.23	36.97	4.19

1) At least partly due to inaccuracies in the subtraction of the protein contaminant.

Methoxyl Groups, Phenolic Hydroxyl Groups and Absorptivities

The contents of phenolic hydroxyl groups (PhOH) and the absorptivities of the lignin samples were determined using UV spectroscopy. All results were corrected for the carbohydrate and protein contents of the samples. The results were also calculated relative to the determined molar mass of the lignins to get the frequencies per aromatic unit (Table VI).

In the alkaline pulps, the methoxyl contents decrease slightly in the order SoAQ>PSAQ>CK. Decreasing methoxyl content can be considered to indicate an increase in catechol structures, which are known to be highly reactive towards

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oxygen delignification. Relative to aromatic units, the content of methoxyl groups is highest in the Si pulp lignin, indicating that demethylation is not a major reaction at least in this type of Si cooking. The same order prevails in the oxygen-delignified pulp lignins, although demethylation compared to the starting pulps is observed. Demethylation is greatest for the CK pulp lignin, inducing the lowest methoxyl content in the CK/O pulp lignin. Similar or even more pronounced demethylation has been reported in the literature (4,9,23). Unlike with the alkaline pulps, oxygen delignification of the Si pulp did not cause demethylation; instead, the content of methylated structures increased during this stage.

The absorptivities of lignin in the alkaline pulps remained unchanged or increased slightly during the oxygen stage. This means that the aliphatic structures formed by oxidation of aromatic units still contain conjugated double bonds, *e.g.* muconic acids, which may be visible in the UV region at 280 nm. Highly UV-active structures, such as quinones, may also contribute to the absorptivity. The absorptivities of the Si pulps are lower because of their larger molar mass. Values 12-14 l/g cm have been reported (22).

The similarity in the PhOH contents of the alkaline pulps is striking. The contents in Table VI have been calculated in three ways to allow proper comparison. For all alkaline pulps, oxygen delignification reduced the frequency of phenolic structures from *ca.* 30% to 20% of all aromatic units. Similar PhOH contents have been reported (19,23). A reduction of up to 50% has been found (9,24), although in some investigations much smaller reductions have been observed (3,25). The variations may be due to the conditions used during oxygen delignification, as has been seen when comparing single and multiple oxygen stages (4). The effect of oxygen delignification on the content of phenolic hydroxyl groups was smaller for the Si pulp.

The analysis method also gives some indication of the type of the PhOH groups. The method is based on the difference in the UV spectrum of lignin induced by ionization of phenolic groups in alkaline solution. Most of the phenols are ionized at pH 12, but some of the structures require strong alkaline solution (0.2N NaOH) to be ionized. The proportion of these weakly acidic phenolic groups can be determined. Another piece of structural information can be obtained from the ionization difference spectrum itself. The ratio of the intensities of the absorbance maxima is dependent on the frequency of α-conjugated (C=C or C=O) phenolic structures, *e.g.* vanillin derivatives. The SoAQ and SoAQ-O pulp lignins contain more weakly acidic phenolic structures than the other alkaline pulps. These may be condensed structures in accordance with the poor bleachabilities of the corresponding pulps. This value is even higher for the Si pulps. The proportion of phenolic structures with α - conjugation was constant at 11 to 12% for the alkaline pulps and 18 to 20% for the alkaline O-delignified pulps, indicating oxidation of the α-position or enrichment or this type of structures. These values were significantly lower for the Si pulps, even after the oxygen stage. An increase in the proportion of α-conjugated structures may reduce the content of weakly acidic structures, as observed in the O-stage lignins, because of their low pK values.

Table VI. Contents of Methoxyl and Phenolic Hydroxyl Groups as well as Absorptivities and Structural Features of the Phenolic Hydroxyl Groups.

	OCH3, weight-%	*OCH3, % of C9*	*Absorptivity of lignin, l/g cm*	*PhOH, mmol/g lignin*	*PhOH, mmol/g a=20-lignin*	*PhOH, % of C9-units*	*Proportion of α-conjugated PhOH, %*	*Proportion of weakly acidic PhOH, %*
CK proRL	12.87	71	20.9	1.85	1.77	32	11	23
PSAQ proRL	13.21	72	21.6	1.89	1.75	32	12	23
SoAQ proRL	13.38	73	21.2	1.90	1.79	32	12	25
Si RL	12.74	79	16.3	1.05	1.29	20	6	28
CK/O proRL	10.95	62	21.3	1.20	1.12	21	18	13
PSAQ/O proRL	11.34	65	21.1	1.13	1.07	20	19	13
SoAQ/O proRL	12.07	68	22.0	1.18	1.07	21	19	16
Si/O NaCl RL	12.93	85	14.4	0.81	1.12	17	8	21

Molar Mass Distribution of Residual Lignin

The molar mass distribution of the isolated lignins showed that molar mass decreases in the order Si>CK>PSAQ>SoAQ for the unbleached pulps. However, only the more soluble proRL fractions could be reliably measured. The less soluble pro is NaOHsP fractions, which probably contain large molecular lignin, were more pronounced in the PSAQ and SoAQ pulps (Fig. 6).

Oxygen delignification reduced the differences between the pulps, and resulted in the CK/O lignin having the lowest molar mass. In this case, the comparison between the pulps is more reliable, because only one lignin fraction was obtained (Fig. 7). The Si pulps both contained very large molecular lignin (Table VII).

Carbohydrate Part of the RLCC Samples

Due to the enzymatic residual lignin isolation method used, the lignins still contained some carbohydrates originating from the lignin-polysaccharide linkages in the corresponding pulp. Therefore, the samples can be regarded as

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Table VII. Molar Masses of the Residual Lignins.

Pulp	*CK*	*PSAQ*	*SoAQ*	*Si*
Unbleached	50900	45100	39900	80000
O-delignified	33000	39100	35900	76400

NOTE The same lignin fractions as in Table VI

residual lignin-carbohydrate complexes (RLCC) rather than pure lignin samples. The content and composition of this carbohydrate residue thus gives information about the chemical and/or physical linkages between these components in the pulp.

The carbohydrate compositions of the RLCCs were corrected for the glucoprotein impurity (13). The protein contents were higher in the RLCCs of the oxygen-delignified alkaline pulp RLCCs than in the unbleached pulp samples (Table VIII). The Si pulp samples were clearly different from the alkaline pulps.

Table VIII. Protein Contents of the RLCCs.

Protein, of the RLCC	*CK*	*PSAQ*	*SoAQ*	*Si*
Unbleached	3.8	3.7	4.0	20.4
Oxygen delignified	9.0	8.5	9.7	5.4 [a)]

a) Si/O NaCl RL

The corrected results are seen in Figure 10a for the RLCCs of the unbleached pulp RLCCs and in Figure 10b for the oxygen stage pulp RLCCs. Carbohydrate compositions were also determined for the corresponding pulps. These results were normalized to the same cellulose content, which was calculated based on the assumption that the ratio of glucose to mannose in glucomannan is 1/5, and that the remaining glucose originates from cellulose (Figure 10c,d). The xylan content was slightly higher in the CK pulp than in other alkaline pulps. This indicates that high xylan content in the pulp does not indicate difficulties in oxygen delignification as has been suggested (26).

Mannose and galactose were the main components of the RLCCs of all alkaline pulps lignins. The proportion of mannose was greatest in the PSAQ RLCC, reflecting the hemicellulose stabilizing conditions of the cooking method. This was also seen in the composition of the pulps. Oxygen delignification changed the proportions of the monosaccharides of the RLCCs only slightly. Galactose, and to a lesser extent, arabinose are enriched in the RLCCs, probably due to linkages between lignin and (arabino)galactans, as has been suggested previously (27). In the Si pulps, lignin seems not to be bound to hemicelluloses to any significant extent; also the protein in these samples might be native rather than adsorbed cellulolytic enzymes. This explains the negative carbohydrate contents obtained after the protein correction. This may also explain why the result is in conflict with that in the literature (22), where linkages to hemicelluloses were suggested.

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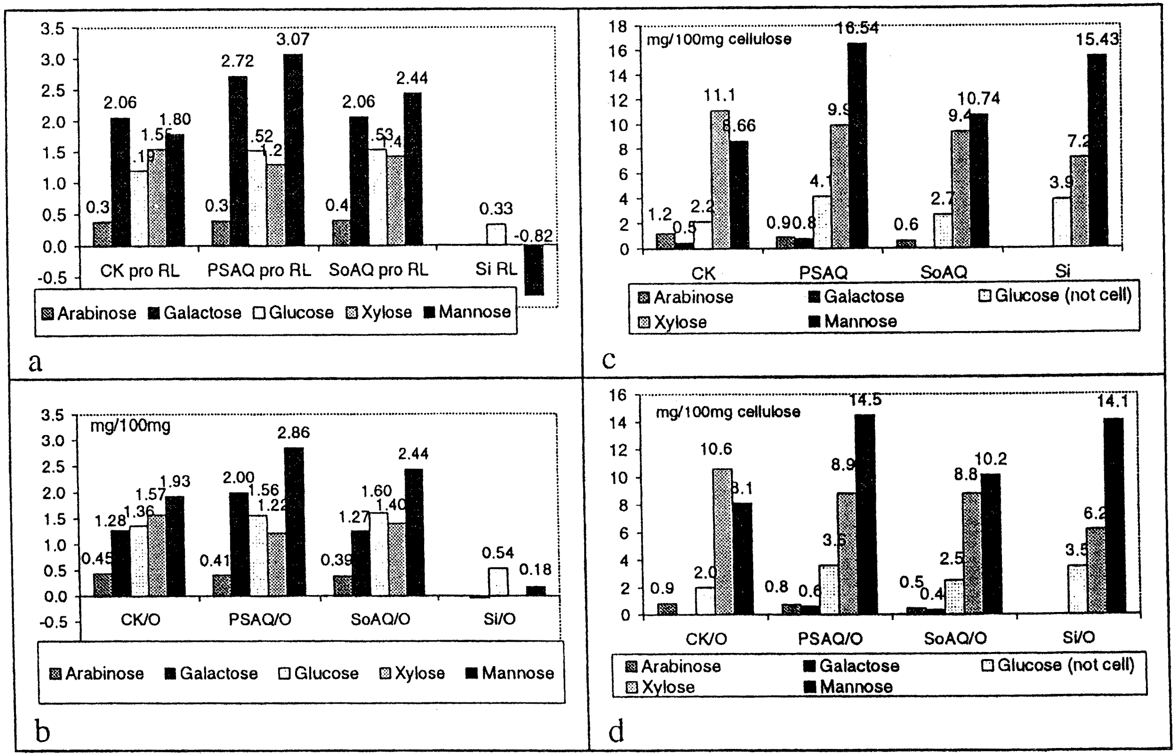

Figure 10. Carbohydrate compositions of the RLCCs (a,b) and the corresponding pulps (c,d). In the pulps, the results have been calculated per 100 mg of cellulose-bound glucose.

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Keeping in mind the striking similarity betwen the structures of the lignin part in these RLCCs, the carbohydrate part might also be related to the differences in the bleachabilities of these pulps. The detailed structure of the oligomers bound to lignin and the type of the linkages may be different in the RLCCs of different pulps. The samples are therefore now being subjected to structural analysis. However, no major difference in the carbohydrate content was found between the unbleached and oxygen delignified pulp RLCCs. As the lignin–bound carbohydrates are believed to represent the lignin-polysaccharide linkages in the corresponding pulps, the result indicates that no formation of new linkages takes place during oxygen delignification.

Conclusions

High yields of protease-purified RLCC were obtained from the alkaline pulps both before and after the oxygen stage. The residual lignins in the Si pulps were so hydrophilic that a modified isolation procedure was developed for them.

The structure of the lignin part of these complexes was analysed by a variety of methods. The effects of oxygen delignification on lignin structure were clearly seen: a reduction in the content of phenolic hydroxyl groups and methoxyl groups, an increase in the proportion of α-conjugated phenolic structures, an increase in carboxylic acid content, a decrease in molar mass and an increase in the hydrophilicity observed during residual lignin isolation.

No major differences in lignin structure connected to the cooking method were found before or after oxygen delignification. However, the degree of demethylation and the decrease in molar mass during oxygen delignification were most pronounced for the CK pulp. This pulp was also the most hydrophilic before oxygen delignification. The CK pulp was also found to have the best bleachability of the alkaline pulps (10). No relevant variation in the content of phenolic hydroxyl groups between the pulp types was found.

The sulfite pulp lignin was structurally quite different from the alkaline pulp lignins and seemed to dissolve during oxygen delignification without any major oxidation reactions.

The carbohydrates in the RLCCs partly reflected the carbohydrate compositions of the corresponding pulps. However, certain carbohydrates were enriched in the RLCCs, indicating linkages between lignin and polysaccharides in the pulps. These, together with the slight differences in the lignin structures, probably affect the bleaching response of these pulps.

Although the alkaline pulp residual lignins were found to be very similar, it must be kept in mind that some structural features which are known to affect bleachability, *e.g.* the contents of enol ether structures, could not be quantified. It should also be kept in mind that the degradative and oxidative reactions of polysaccharides, not only lignin, consume chemicals (*e.g.* alkali in oxygen delignification). Thus, even differences in the hemicellulose composition and stability affect pulp bleachability.

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Osmo Pekkala and Marjatta Ranua are thanked for their expertise concerning the preparation of the pulps.

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Chapter 5

Breaking the Oxygen Delignification Barrier: Lignin Reactivity and Inactivity

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Low kappa factors (0.05 KF) of chlorine and chlorine dioxide were employed to chemically pretreat softwood kraft pulp and associated residual lignin before an oxygen delignification stage. Quasi-pretreatments using nitrogen dioxide were performed in parallel and all results were compared to high kappa factor treatments (KF = 0.20) of pulp and lignin to exaggerate and examine the lignin structural changes contributing to the pulp delignification response during an oxygen stage. The principal spectroscopic method chosen to investigate the chemical changes in lignin was ^{31}P NMR. One of the most significant results arising from these studies was the relatively constant content (< 30% change) of the condensed phenolics despite the efficacy of the chemical pretreatment stage for the lignins. Interestingly, the free phenolics were not appreciably consumed, strongly suggesting that these moieties should be the focus of any future attempts to maximize the performance of an oxygen stage.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
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The issue of overcoming the limits to oxygen delignification has received increased research attention recently. Since oxygen provides significant environmental and economic benefits, reportedly being able to increase yield, successfully reduce lignin levels and thus provide bleaching cost savings while maintaining compatibility with recovery operations, increased delignification without compromising yield or pulp properties is a very desirable goal [1-10]. One promising avenue for enhanced delignification that has witnessed considerable research attention is the use of pretreatments to improve the bleaching response of pulp in an oxygen stage [11-13]. Pretreatments may be defined as low kappa factor (KF, low molecular chlorine multiples/kappa of pulp) pulp bleaching stages that follow pulping to increase pulp bleachability without adversely affecting pulp properties.

The current research was conducted to elucidate the chemical basis for the limits in oxygen delignification through an analysis of various significant functional groups in lignin. These groups were analyzed after chemical treatments that employed low and high kappa factors of chlorine and chlorine dioxide in addition to an intermediate kappa factor of nitrogen dioxide. Gierer and others have provided the fundamental chemical underpinnings for the structural changes imparted to lignin during oxygen delignification [14-16]. Highly reactive hydroxyl radicals, for example, can react with aromatic and aliphatic lignin structures to generate organic radicals which are purportedly susceptible to attack by superoxide. Superoxide has been suggested to be involved in the scission of aromatic, conjugated, and aliphatic (side chains) lignin structures. Ring opening and side chain elimination reactions can induce carboxylic acid formation and enhance lignin solubility in alkaline conditions. The resistance of lignin removal after 50% delignification during an oxygen stage has been extensively studied and has in part been attributed to carbon-carbon bond structures that are recalcitrant to degradation. For example, dimeric arylpropane units containing saturated side chains such as bicreosol display a reduced reactivity in oxygen systems [17]. In addition, while Lai reports that diphenylmethane lignin units are notoriously stable, he contends that condensed phenolic lignin structures are unstable, while Argyropoulos has maintained that condensed phenolic structures are the major factor limiting oxygen delignification [18-24]. Much of the work that remains to be done in this area requires an increased understanding of the role and fate of the lignin structures that are activated or that remain inert during an oxygen stage.

Herein, we report the structural changes that occur to the residual kraft lignin of two mill pulps, manufactured by EMCC® (extended modified kraft cooking) and CC (conventional cooking) technologies, after both chemical pretreatments (using varying kappa factor charges of chlorine, chlorine dioxide, and nitrogen dioxide) and followed by oxygen delignification. Specifically we have

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correlated the chemical structural changes of the lignin to the delignification efficiency observed for the two industrial pulps. We have obtained quantitative ^{31}P NMR spectra and elemental analyses of all lignins in an effort to identify the structural factors responsible for the inactivity and reactivity of lignin during oxygen delignification.

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Methods

Pulping and Oxygen Delignification. Kraft pulps were manufactured by industrial sponsors using typical EMCC® (MK) and Conventional Cooking (CK) technologies. The kappa numbers measured for the MK and CK pulps were 23.2 and 22.4, respectively.

Oxygen delignification runs were conducted on a 300-ml PARR Instruments Pressure Reactor employing the following conditions: 60 minutes, 100°C, 100 psi, 2.33% NaOH charge (relative to mass of pulp), 10% consistency (when applicable), and subject to mild (5 hz) impeller-blade stirring. The headspace in the PARR reactor was thoroughly flushed with oxygen before application of pressure. Lignin oxygen runs were done by dissolving 75 mg of each lignin into 60 mL of alkaline charged water and stirred. The starting and ending pHs for the runs ranged from 11.3 to approximately 10.. All pulps and lignins were consequently removed after the runs and allowed to cool before either a thorough distilled water wash or acid precipitation, respectively.

Lignin Isolation. The residual lignins of the MK and CK pulps were isolated by a slightly modified acid hydrolysis procedure that involved a 1 hour reflux of the pulps in an 0.1 N HCl solution containing 9:1 *p*-dioxane:water for. *P*-dioxane was distilled over sodium borohydride powder for one hour immediately before use. After reflux, the supernatant was collected and the dioxane was removed under reduced pressure. The pH of the remaining water suspension was adjusted to 2.0; the resultant lignin suspension was frozen to increase lignin coalescence, and allowed to thaw after 24 hours The precipitate was collected by centrifugation. The preceding process was repeated in triplicate using fresh water rinses of pH = 2.0. The resultant lignin was lyophilized and collected. Product recovery yields were based on the pulp starting kappas (total lignin content) and typically ranged from approximately 40 to 60 %.

High, Intermediate, and Low Kappa Factor Pretreatments. The pulp pretreatments were conducted at 0.05 kappa factor using freshly prepared chlorine or chlorine dioxide. In order to amplify and more fully explain the chemical effects induced by these pretreatments, higher kappa factors of 0.20 were used, in addition to the use of an intermediate kappa factor of 0.10 for a nitrogen dioxide pretreatment. Nitrogen dioxide pretreatments were accomplished by introducing the appropriate amount of a sodium nitrite solution followed by nitric acid. All pulp pretreatments were conducted in sealed bags and were run at 70° C for 30 minutes while maintaining a final pH of approximately 2.0. All pretreatment conditions used for the pulps were applied to the residual lignins, except that the lignin was dissolved in 9:1 dioxane:water and stirred at ambient temperature in round bottom flasks. All kappa values have an experimental error of approximately 3%. Bleachability in this work will refer to a given level of delignification at constant conditions (caustic, temperature, and time of reaction).

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Recovery of Lignin from Pretreatment and Oxygen Bleaching. Lignin pretreatment and oxygen bleaching runs were performed after the pulp runs to provide a fundamental basis for the delignification observed in the pulps through analysis of discrete lignin functional groups . Thus, the residual lignins from the MK and CK pulps were systematically isolated and investigated through ^{31}P NMR. Since our objective was to provide an accurate summary of the fate of the lignin structural subunits after pretreatment and a subsequent oxygen stage, it was necessary to recover as much of lignin as possible after pretreatment and oxygen bleaching. All lignins were therefore recovered as quantitatively as possible by exhaustive ethyl acetate extraction as described by Asgari and Argyropoulos [22]. Any remaining aqueous phase that remained after chemical reactions in either dioxane/water or alkaline water was removed under reduced pressure and DMF was added to the remaining precipitate to specifically dissolve low molecular weight lignin fragments [22]. The DMF layerwas filtered to remove insoluble salts and the remaining solution was added drop-wise to diethyl ether to precipitate the lignin. Lignins were lyophilized and vacuum-oven dried before NMR analyses. Recovery yields for the lignins after both pretreatment and oxygen bleaching runs typically ranged from 55-75%.

Quantitative Lignin ^{31}P NMR Analyses. Spectral characterization of the residual lignins was accomplished on a 400 MHz Bruker DX Spectrometer employing published procedures [25]. All residual lignins were dissolved in a solution of 650 μL of pyridine/$CDCl_3$ (v/v 1.6/1) that contained either cyclohexanol or *endo*-N-hydroxy-5-norbornene-2,3-dicarboximide as an internal standard and chromium acetylacetonate as an internal relaxation agent. The samples were phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. Regions for integration have been reported elsewhere [26]. The integration values have a reproducibility of approximately 95%.

Results and Discussion

Pulp Studies

The initial studies focused on determining the effect of pulp pretreatments on the delignification responses of MK and CK pulps. Shown in Figure 1 are the delignification responses of the pulps after oxygen delignification.

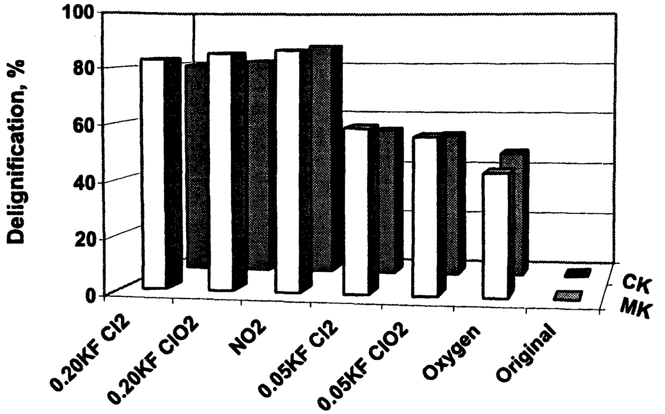

Figure 1. The levels of oxygen delignification obtained for the MK and CK pulps as a function of pretreatment.

The oxygen control level of delignification achieved for both pulps in is approximately 45%. The low KF chlorine and chlorine dioxide pretreatments increase the delignification by an additional 30% over the threshold levels, and additionally the MK pulp has a better delignification response than the CK pulp by over 2 kappa units. One of the more remarkable findings in the above figure is the high delignification achievable by a relatively modest NO_2 pretreatment. Obviously, modest NO_2 treatments (KF = 0.10) tremendously boost delignification in a subsequent oxygen stage in a manner comparable to high kappa factor pretreatments using chlorine and chlorine dioxide (KF = 0.20). The enhancement of the performance of an oxygen stage by varying concentrations of NO_2 has been well described in the literature [27-29]. These results validate the ability of this particular treatment to predispose lignin to enhanced oxygen delignification. It is noteworthy that this data provides evidence that the MK pulps display slightly better bleachability than the CK pulps (on the order of at least 10%).

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Carboxylic Acid Content

The carboxylic acid group is typically associated with a significant increase in the oxidation state of lignin. It is a important structural change that occurs in any bleaching process since it the primary way of imparting an enhanced degree of solubility to lignin and typically follows ring opening, aliphatic cleavage, or other oxidative fragmentation of lignin. Shown in Table 1 is a list of the carboxylic acid group changes for all the lignins analyzed in this study. A chlorine dioxide (D) pretreatment caused approximately 30% increase in the overall acid content of both the CK and MK lignins, not unlike what has been previously observed in D bleaching [30]. However, chlorine (C) pretreatments did not induce the generation of similar acid levels.

Table 1. The carboxylic acid content for pretreated and post-oxygen (expressed as pretreatment/KF/O) treatment residual MK and CK lignins expressed in mmoles/gram of lignin. The recovery yields for these lignins ranged between 55 and 75% of the original lignin mass.

CARBOXYLIC ACID CONTENT	MK (mmoles/g lignin)	CK
Brown Stock	0.30	0.31
Oxygen Control	0.76	0.90
Cl_2/0.05	0.24	0.26
Cl_2/0.05/O	0.70	0.80
ClO_2/0.05	0.41	0.43
ClO_2/0.05/O	1.04	1.01
NO_2/0.10	0.24	0.22
NO_2/0.10/O	0.76	0.58
Cl_2/0.20	0.21	0.25
Cl_2/0.20/O	0.51	0.54
ClO_2/0.20	0.72	0.65
ClO_2/0.20/O	1.39	1.09

We found from elemental analysis, however, that there was a heavy incorporation of chlorine, up to 15% in the lignins, which partially offset the introduction of acid groups. Also, it was found by Lachenal et al. that at low KFs of chlorine, acid level increases are not as appreciable as for a D pretreatment [31]. In fact, the generation of carbon dioxide and carbonate have been found to be significant pathways during chlorinations of pulp [31]. Interestingly, carboxylic acid generation was not found to be as significant in NO_2 pretreatments. Again, this was not surprising since significant incorporation of nitrogen (approximately 5-10%) was found and a previously described mechanism of NO_2 reactions with lignin as shown in Figure 2 provides a partial explanation [29]. NO_2 has been postulated by Walding et al. to induce significant depolymerization through nitration reactions. In fact, Samuelson has found that the tendency to delignify from such treatments owes not so much to increased hydrophilicity by acid incorporation, but because of extensive lignin degradation into fragments smaller than found in ordinary bleaching sequences.

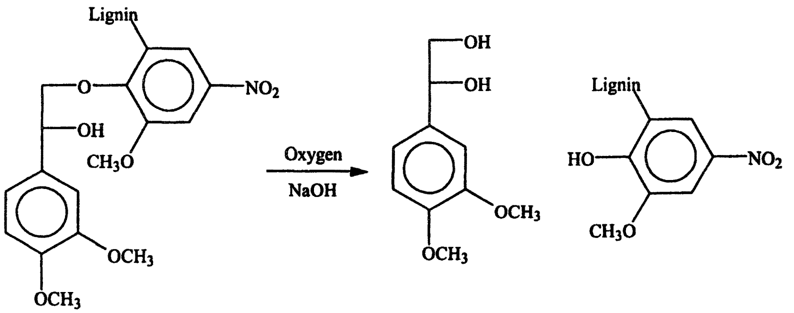

Figure 2. The hydrolysis of nitrated lignin during an oxygen stage that follows a NO_2 pretreatment as described in Walding's work is shown above. It is expected to be facile since the highly electronegative nitrated lignin subunit can be displaced by the α-hydroxyl group under alkaline conditions.

The discrepancy between the levels of acid between CK and MK (higher for MK, opposed to trends shown in Table 1) after an oxygen stage can be explained by the greater abundance of aliphatic hydroxyls in MK over CK by more than 10%. The α-hydroxyls can participate in a base-induced intramolecular expulsion of an adjoining β-nitrated ring leaving a vicinal diol that can further oxidize to a terminal acid during oxygen delignification

The control oxygen lignins demonstrated a 2-3 fold increase in acid levels and the CK lignin had approximately 15% higher levels. The D pretreated MK lignin shows an enhanced carboxylic acid content beyond what is observed for the oxygen control that is consistent with the slightly better bleachability of its associated pulp (see Figure 1). Employing a high KF pretreatment before

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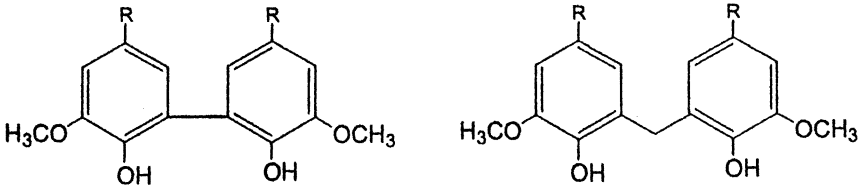
oxygen exaggerates the acid differences between the starting and oxygen treated lignins consistent with the pulp data. An important difference in lignin structure to account for the slightly better bleachability of the MK over CK are the higher levels of condensed phenolics and aliphatic hydroxyls of the MK lignin. The phenolics are primary sites of reactivity for chlorine dioxide and the increased levels in the MK may explain the increased delignification response of the MK pulp.

Phenolic Content

Lignin contains both condensed and non-condensed (free) phenolic lignin structures which have tremendous importance in the overall response of lignin to oxidants. Several of the structures of these important functionalities which comprise the focus of our NMR investigations are shown in Figure 3. The salient difference between condensed and non-condensed structures is the substitution pattern at the 5-position of two arylpropanoid units.

5-Condensed Phenolic Subunits of Lignin



Non-condensed (free) Phenolic Subunits of Lignin

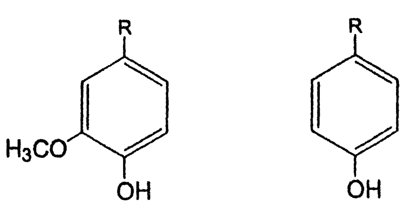


Figure 3. The structures of some typical condensed and non-condensed (free) phenolic units in residual kraft lignins are displayed. Notice that the salient differences occur in the substitution pattern at the 5-carbon of the lignin aromatic subunits, where condensed structures having C-C bonds, whereas non-condensed have C-H bonds.

Remarkably, although C pretreatments (both high and low KFs) did not increase carboxylic acid levels, they nonetheless tended to afford higher levels of condensed phenolics. Shown in Figure 4 are the actual levels of the condensed phenolics for the pretreated lignins. Table 2 provides a comparative analysis of the phenolic levels of all of the lignins. The levels of condensed phenolics in the C pretreatment are more significant than observed in the D pretreatment. This suggests that one of the potential side reactions of a C pretreatment is the formation of coupling products by radical reactions [31, 32]. Nonetheless, this pretreatment does not prevent the delignification efficiency associated with a subsequent oxygen stage.

Table 2. The non-condensed and 5-condensed phenolic content for the pretreated and post-oxygen (italics) residual MK and CK lignins expressed in mmoles/gram of lignin.

PHENOLIC CONTENT	**MK (mmoles/g lignin)**		**CK**	
	Non-Condensed	**Condensed**	**Non-Condensed**	**Condensed**
Brown Stock	0.93	0.89	0.93	0.82
Oxygen Control	0.60	0.64	0.69	0.68
Cl_2/0.05	0.67	0.90	0.64	0.86
Cl_2/0.05/Oxygen	0.44	0.63	0.46	0.69
ClO_2/0.05	0.68	0.69	0.73	0.70
ClO_2/0.05/Oxygen	0.53	0.58	0.56	0.67
NO_2/0.10	0.20	0.43	0.19	0.44
NO_2/0.10/Oxygen	0.30	0.52	0.24	0.46
Cl_2/0.20	0.29	0.69	0.28	0.77
Cl_2/0.20/Oxygen	0.27	0.58	0.30	0.67
ClO_2/0.20	0.36	0.54	0.28	0.51
ClO_2/0.20/Oxygen	0.34	0.46	0.40	0.35

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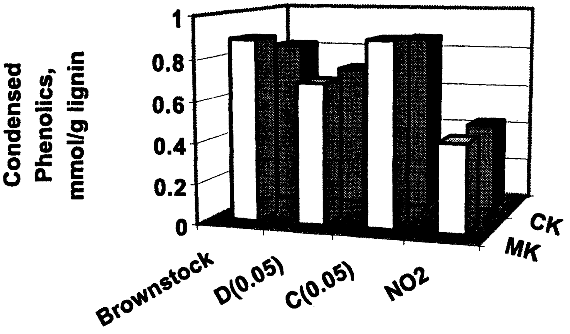

Figure 4. The content of condensed phenolics in the pretreated residual MK and CK lignins.

An experimental result that provides insight for the level of bleachability is the level of carboxylic acid for the lignins after a control oxygen stage. The CK lignin has a larger carboxylic acid content than the MK lignin, and is in fact more bleachable as a pulp. The MK lignin, however, has more condensed phenolic structures than the CK that may partially explain its diminished bleachability, a cogent argument that has been the subject of numerous investigations [20, 22, 23, 36-38]. Yet, a C/O stage appears to refute this latter argument since the MK pulp is slightly more bleachable, although it has a higher level of condensed structures. This slight increase in reactivity may well be due to other structural features such as the aliphatic hydroxyl groups (*vide infra*).

Condensed phenolic structures have received considerable attention as a major source that contribute to the inactivity of lignin during oxygen delignification. The current work has examined their role in the context of a preactivation step (pretreatments) of the lignin before oxygen and found that they remain relatively intransigent throughout the chemical bleaching/oxygen steps. Shown in Figure 5 are the levels of condensed phenolics for the lignins after an oxygen stage.

The condensed levels do not vary more than 30% throughout. *Moreover, the delignification efficiency observed in the pulps is not compromised as a result of these structural components.* This data strongly indicates that these structures are robust and therefore stable, *and are not intrinsically the primary constituents that limit the reactivity of lignin.*

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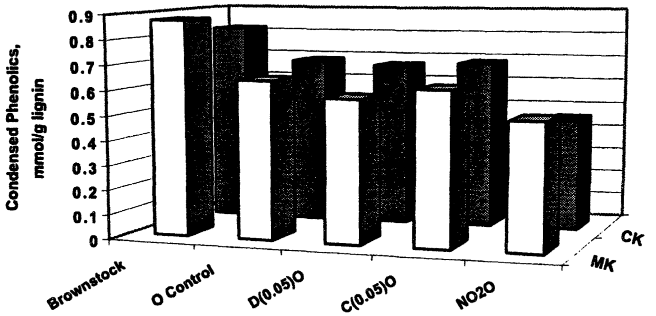

Figure 5. The content of condensed phenolics in the post-oxygen stage residual MK and CK lignins.

In fact, even high kappa factor pretreatments do not significantly affect their relative distribution with respect to total phenolics as evidenced in Figure 6. In light of the previous data, Figure 6 supports the observation that condensed phenolics are not depleted as much as free phenolics, yet, free phenolics do not change dramatically enough to account for the delignification observed in the pulps. In fact, the pretreatment phenolic ratios are quite constant, testifying to the relative robustness of both the condensed and non-condensed phenolic contents.

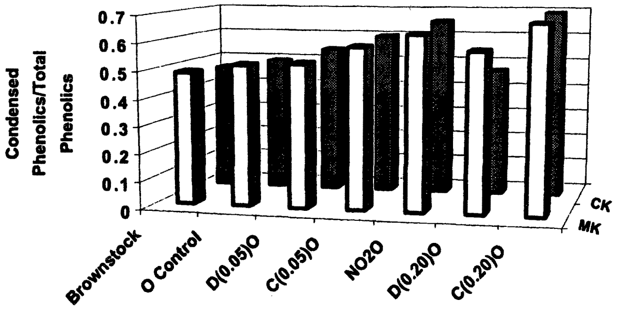

Figure 6. The ratio of condensed phenolics content to total phenolic content in the post-oxygen stage residual MK and CK lignins.

Again, the non-condensed phenolics are surprisingly inefficiently consumed. Most work strongly indicates that these are the primary reactive sites for D and

O stages [32, 33]. As shown in Table 2 (*vide supra*), the relative change in the non-condensed phenolics as part of a pretreatment is not appreciable with regard to the bleachability results for the pulps. This result suggests that the phenolic sites are not being consumed as would be expected to account for the decrease in lignin content and that alternative explanations may account for these drops. Perhaps lignin exhibits phenolic sites of differing reactivity based on their electrochemical potentials and environmental constraints and sites that contribute to increased oxidation/solubilization are activated by a pretreatment.

Aliphatics

As shown in Figure 7, the aliphatic levels for the pretreated lignins diminish slightly, but this is not unusual considering that most of the pretreatments do not attack the aliphatic side chains appreciably. Chlorine, however, is known to attack side chains and efficiently deplete this functionality which is demonstrated in the pretreatment. Interestingly, although MK has a greater proportion of condensed structures, suggesting that its bleachability is hindered, it is nevertheless more bleachable than CK, perhaps as a result of the disparity in aliphatic levels between the two lignins. An exaggerated high kappa factor C pretreatment, moreover, extensively consumes the aliphatics, further supporting the latter argument for the heightened bleachability of MK versus CK pulps.

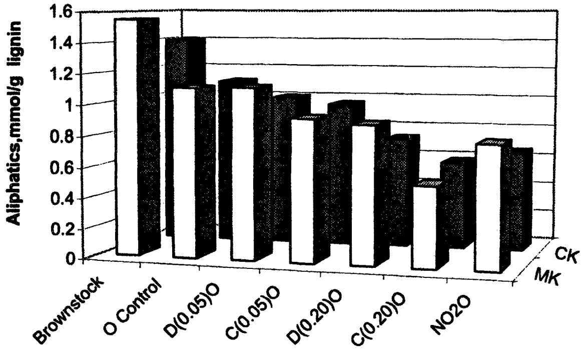

Figure 7. The content of aliphatic hydroxyl functionalities in the pretreated and post-oxygen stage residual MK and CK lignins.

Conclusions

The free phenolics of residual lignin are surprisingly not appreciably consumed in an oxygen bleaching stage following a pretreatment stage of

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chlorine, chlorine dioxide, or nitrogen dioxide despite the enhanced bleachability of pretreated pulps. The NMR data strongly implies that more than 50% of these units are resistant to oxidation, while the concentration of condensed phenolics remain relatively constant. The major difference between the MK and CK lignins are the higher levels of condensed phenolics in the MK lignin which may partially assist during lignin oxidation for the C and D pretreatments. The MK pulps are slightly easier to bleach and the NO_2 pretreatment was extremely effective in promoting the bleachability of the pulps, which may be a consequence of its ability to fragment lignin efficiently via nitration. Condensed phenolics are nonetheless quite resistant to degradation and appear to remain in the lignin samples despite the pretreatments. Their relative robustness does not, however, appear to be the main rationale for the inactivity of lignin toward oxygen delignification, but serves to suggest that the nature and reactivity of the free phenolics deserve increasing scrutiny.

Acknowledgements

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Chapter 6

The Reactions of Peroxides with Lignin and Lignin Model Compounds

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The reactions of peroxides with lignin and lignin model compounds have been extensively investigated. It is generally accepted that peroxides react via two reaction mechanisms depending on the structure of the peroxide and the reaction conditions employed. In alkaline conditions peroxides react nucleophilically with electron deficient carbonyl and conjugated carbonyl structures. In neutral to acidic media, most peroxides react with electron rich aromatic and olefinic structures via electrophilic pathways. In this paper, the reactions of peroxides with lignocellulosic materials, concentrating on lignin and lignin model compounds are reviewed. Emphasis is placed on the reactions of hydrogen peroxide and peroxy acids. However, owing to their chemical structures, and susceptibility to thermal and transition metal catalyzed decomposition, the reactions of hydroxyl, HO· and superoxide $\cdot O_2^-$ radicals with lignin model compounds will also be discussed.

With an ever-increasing demand by governmental as well as environmental agencies for environmentally benign bleaching technology, the pulp and paper industry is being forced to reduce the amount of chlorine containing bleaching reagents it employs. Processes utilizing peroxygen-based reagents such as hydrogen peroxide and peroxy acids are thus being implemented. As a result, an exorbitant amount of literature exists on the chemistry of peroxides and lignin model systems under the conditions utilized during the bleaching of chemical pulps (*1-14*).

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Traditionally, hydrogen peroxide has been classified as a lignin-preserving bleaching reagent. It preferentially removes chromophoric structures present in residual lignin, but is incapable of degrading the lignin network (*14-17*). To degrade and remove the residual lignin, hydrogen peroxide must be activated. Conventionally this is accomplished via the in situ formation of a peroxy acid by the addition of acetic, formic or sulfuric acid or cyanamide into a hydrogen peroxide solution (*5,18-28*). The peroxy acid then reacts as an electrophile with lignin, leading to oxidation and subsequent degradation (*29-34*). Similarly the use of metalloporphyrins (*35-41*) and other biomimetic systems (*42-47*) have been explored as means to increase the reactivity of hydrogen peroxide. In the following chapter, a review of the recent literature of hydrogen peroxide, and peroxy-acids in aqueous solutions will be given. Emphasis will be on the oxidation of lignin model compounds, lignin preparations and pulp. In addition, the reactions of the oxygen-based radicals generated in these peroxide systems with similar materials will be mentioned.

Properties of Aqueous Peroxide Solutions

An important aspect of hydroperoxides and peroxy acids is the acidity of the peroxide proton. Hydroperoxides, ROOH, are generally stronger acids than the corresponding alcohols, ROH (*48*). This shift in pK_a has been attributed to (i) the RO group which has a greater electronegativity than the R group, causes a relative decrease in the electron density on the O-H bond in ROOH as compared to ROH and (ii) the greater the electron withdraw by RO more effectively stabilizes the resulting peroxy anion. On the other hand, peroxy acids are generally weaker than their parent acids (*49*). Two plausible explanations exist, (i) intramolecular hydrogen bonding in the peroxy acid stabilizes the neutral form relative to the anion, (ii) the parent acid has a resonance stabilized anion form making the acid relatively strong, whereas the peroxy anion has only the inductively delocalized electronic configuration. When undissociated, hydroperoxides and peroxy acids are relatively stable.

$$RO_2H + HO^- \rightleftharpoons ROO^- + H_2O \qquad (1)$$

Peroxides are powerful oxidizing agents reacting as both electrophiles and nucleophiles. In nucleophilic reactions, it is generally accepted that the conjugate base, peroxyl anion, is the active species, since conditions for its formation parallel those required for attaining a maximum reaction rate (*50,51*). Due to its chemical structure, the peroxyl anion (ROO^-) is a strong nucleophile. This enhanced nucleophilicity is characteristic of molecules with unshared pair of electrons on the atom adjacent, or alpha, to the nucleophilic

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atom, and is known as the alpha effect (*52-54*). As a result, peroxides are modest two-electron transfer agents (*23,55-58*). In alkaline conditions, the primary reaction is alkaline or nucleophilic epoxidation involving electron deficient alkenes (*59*). Depending on the peroxide used, the stereospecificity of the epoxidation will differ (*60*). In general stereospecificity increases (i) with increasing nucleofugacity (i.e. leaving group ability) of the peroxide, and (ii) with decreasing electron-deficiency of the alkene.

The most characteristic electrophilic reactions of peroxides are oxygen transfer reactions, particularly epoxidation (*61-63*) and hydroxylation reactions (*64-71*). The oxygen atoms of the peroxides are transferred to substrate nucleophiles with lone-pair or π-bonding electrons. It has been shown that ionic intermediates are not present, and that the transition state involves the coordination of the π-electrons with the peroxide-oxygen. Again, peroxide and olefin structure dramatically effect the reactions. Both electron-donating substituents on the alkene/aromatic ring and electron withdrawing groups on the peroxide accelerate the reactions. Peroxy acids show enhanced reactivity relative to hydroperoxides due to their increased nucleofugacity, which is apparent from the acidities of the departing acids (pK_a~4-5) relative to those of alcohols (pK_a~15) and increased electrophilicity (*72*).

Thermodynamically, peroxides are potentially unstable, decomposing exothermically according to equation 2.

$$\mathrm{ROOH} \longrightarrow \mathrm{ROH} + {}^{1}/_{2}\,\mathrm{O_2} \qquad (2)$$

The facile decomposition is a result of the weak O-O bonds, ~ 31 kcal mol^{-1} for $CH_3C(O)O\text{-}OH$ vs 51 kcal mol^{-1} for HO-OH, which are easily cleaved by light and heat (*73*). In both peroxy acids and hydroperoxides, heating above a critical temperature (peracetic acid ~80°C, H_2O_2 ~120°C) initiates homolysis of the O-O bond, leading to the formation of radical species. What ensues is the kinetic decomposition of the peroxide to a variety of radical intermediates. Fortunately, the activation energy of this decomposition is high and not readily reached in aqueous solutions (*74*). The decomposition is however easily catalyzed by trace amounts of transition metal ions and other easily oxidizable materials.

It is well established that peroxides undergo facile alkaline decomposition in which the rate of decomposition increases with increasing pH and hydroxide ion concentration (*75-77*). In hydrogen peroxide, this decomposition proceeds via a disproportionation reaction, with a maximum rate at the pH of its pK_a (11.6 @ 25 °C) (*48*).

$$\mathrm{HOOH} + \mathrm{HOO^-} \longrightarrow \mathrm{HO^{\bullet}} + \mathrm{HOO^{\bullet}} + \mathrm{HO^-} \qquad (3)$$

$$\mathrm{HOOH} + \mathrm{HOO^-} \longrightarrow \mathrm{HO^-} + {}^{3}\mathrm{O_2}\,({}^{1}\mathrm{O_2}) + \mathrm{H_2O} \qquad (4)$$

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Disproportionation then leads to a rapid decomposition of hydrogen peroxide through an autocatalytic process. In aqueous media, the kinetics and thermodynamics of a number of "*active oxygen*" species have been evaluated by pulse radiolysis (*78-80*).

$$HOO^- + HO\cdot \longrightarrow \cdot O_2^- + H_2O \qquad k = 3x10^7\ M^{-1}s^{-1} \qquad (5)$$

$$HOOH + HO\cdot \longrightarrow HOO\cdot + H_2O \qquad k = 3x10^7\ M^{-1}s^{-1} \qquad (6)$$

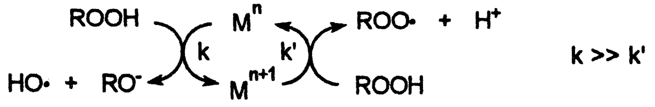
$$HOO\cdot \rightleftharpoons \cdot O_2^- + H^+ \qquad pKa = 4.8\ @\ 25^oC \qquad (7)$$

$$HO\cdot + \cdot O_2^- \longrightarrow HO^- + O_2 \qquad k = 1x10^{10}\ M^{-1}s^{-1} \qquad (8)$$

$$HOOH + \cdot O_2^- \longrightarrow HO\cdot + HO^- + O_2 \qquad k = 1x10^{-1}\ M^{-1}s^{-1} \qquad (9)$$

From the above information, reactions 3, 5, 6 and 8 appear to represent a viable mechanism for the base-induced decomposition of hydrogen peroxide to oxygen and water. A similar series of reactions occurs in peroxy acids in aqueous conditions. In addition, as hydrogen peroxide is also produced in the alkaline hydrolysis of peroxy acids, the series of reactions outlined above will also occur.

A kinetic chain reaction can be catalyzed by traces (10-20 ppm) of transition metal ions, particularly iron, cobalt, manganese and copper, and is often referred to as Fenton's chemistry (*81,82*). In such conditions the peroxide acts as both a reducing and oxidizing reagent with the transition metal ions in the higher and lower valence states respectively.



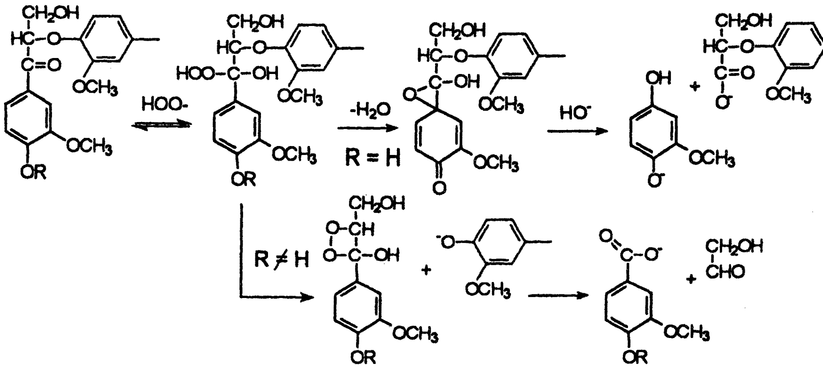
As these transition metal ions are insoluble under alkaline conditions, a heterogeneous surface-catalyzed reaction caused by colloidal transition metal oxides/hydroxides has been proposed (*83*). To minimize the effect of these transition metal induced decomposition reactions, many techniques have been employed, which include the addition of sequestering agents (*84-88*), and/or inorganic salts, (*89-91*) as well as acid washing (*92-94*) or other pretreatments (*95-103*) to remove the majority of these metal ion contaminants prior to peroxide bleaching (*104-107*).

Thus, peroxygen bleaching involves a series of oxygen containing compounds that are formed and consumed dependent on pH, temperature and organic/inorganic contaminants. Therefore, depending on which of these '*active oxygen*' compounds are present, the outcome and extent of lignin degradation will vary (*1*).

Reactions of hydrogen peroxide with lignin model systems

Under the conditions representing those used for commercial chemical pulp bleaching, alkaline hydrogen peroxide has been shown to react with both aliphatic and aromatic structures in lignin (*108-117*). However, the majority of the work in this area and the conclusions drawn therein has been a result of reactions of hydrogen peroxide degradation products, i.e. oxidative radicals, and not hydrogen peroxide itself (*116*). Fortunately, through the careful control of reaction conditions, pH, temperature, metal management etc., the predominant reaction pathways of alkaline hydrogen peroxide with lignins have been ascertained.

Prevalent functionalities in residual lignins are the α-carbinol and α-carbonyl structures (*118*). In the degradation of aryl-α-carbonyl structures, both free and etherified phenolic hydroxyl compounds undergo oxidation by alkaline hydrogen peroxide (*119,120*). The initial step is nucleophilic attack of hydrogen peroxide at the α-carbonyl carbon. In the presence of a free-phenolic hydroxyl compound R=H, the reaction proceeds by way of the Dakin-reaction (*121*). This reaction involves, as a rate-determining step, the formation of an intermediate epoxide, which under alkaline conditions is rapidly hydrolyzed accompanied by cleavage of the C_1-C_α bond.



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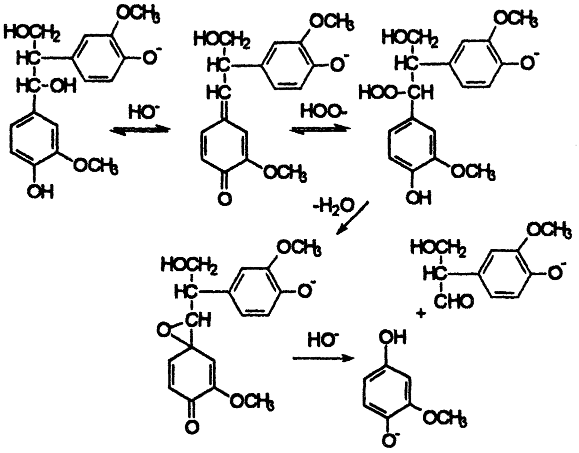
In the absence of a free-phenolic hydroxyl group, the initially formed hydroperoxide is precluded from undergoing the Dakin reaction. Instead the hydroperoxide undergoes intramolecular nucleophilic attack leading to the formation of a new phenolic hydroxyl group and a dioxetane intermediate, which subsequently decomposes to the corresponding benzaldehyde, which is susceptible to further oxidation (*122*). It has been reported that in the decomposition of such β-aryl ethers, the free-phenolic compounds react an order of magnitude faster than the etherified counterpart (*113,123*).

For α-carbinol structures, whether β-aryl ethers, β-1 or β-5 diols, only free-phenolic lignin moieties react with alkaline hydrogen peroxide, even at extreme reaction conditions (*122,124*). The reaction proceeds by way of a quinone methide intermediate followed by the Dakin-like reaction.



In this reaction, the quinone methide intermediate rapidly reacts with the hydroperoxyl anion to produce the corresponding hydroperoxide. It then rearranges in a Dakin reaction like fashion, resulting in the cleavage of the C_1-C_α bond.

Compared to the Dakin reaction, the Dakin-like reaction is extremely slow and does not occur at any appreciable rate at temperatures below 50°C (*113,122,125*). This decreased reactivity is likely due to the decreased rate of formation of the necessary quinone methide intermediate. As a result the corresponding hydroperoxide is not formed, thereby making side-chain displacement not possible. Although the predominant reaction of α-carbinol containing phenolic compounds is the Dakin-like reaction, numerous investigators have reported that the oxidation to the corresponding α-carbonyl compound also takes place (*126,127*). However we have recently shown that in the absence of peroxide decomposition, this oxidation does not occur, even at extreme temperatures (*123*).

By suppressing thermal and metal induced decomposition of hydrogen peroxide, the reactions of lignin and lignin model compounds can be studied at temperatures higher then those previously employed (*123,124*). Thus, in the presence of DTMPA (diethylene-triamine-pentamethylene phosphonic acid) hydrogen peroxide was found to be completely stable at 90°C under peroxide bleaching conditions. While reaction pathways remained essentially the same, the rate of some reactions greatly increased when the reaction temperature was increased from 50°C to 90°C. For example, apocynol, which reacts exclusively via the Dakin-like reaction, was almost completely degraded after 1 hour at 90°C, whereas only a fraction was reacted at 50°C. Similarly, several nonphenolic lignin structures, known to be stable to alkaline hydrogen peroxide underwent oxidation to varying degrees.

Other lignin structures containing carbonyl and ethylenic groups in the side chain have also been studied (*110,128*). In these systems peroxide oxidation occurs via conjugate addition to form the corresponding oxirane followed by hydrolysis and carbon-carbon bond cleavage.

Finally, extended carbonyl structures such as quinoids play an important role in the oxidation of phenolic lignin model compounds, and likely residual lignin, by alkaline hydrogen peroxide. Recall that quinone methides enable side-chain displacement in the Dakin-like reaction (discussed above). Para- and ortho-quinoids, which constitute chromophoric structures found in residual lignins, are also rapidly degraded by alkaline hydrogen peroxide. Primarily produced in the electrophilic reactions of previous bleaching stages, or during peroxide bleaching in which peroxide decomposition has occurred, these simple quinoid-type chromophoric structures are rapidly degraded to mono/di functional carboxylic acids (*129-132*). *O-* and *p*-quinoid rings are comprised of dual enone structures offering multiple sites for attack by hydroperoxyl anion (*133*). Para-quinoids reactions start through nucleophilic attack of one of the electron deficient carbon atoms in the quinoid ring by hydroperoxyl anions, followed by either elimination of a hydroxyl ion (formation of an epoxide) or ring closure to a dioxetane structure. These intermediates are then further attacked by perhydroxyl and hydroxyl anions in one or several steps to yield the final carboxylic acid products.

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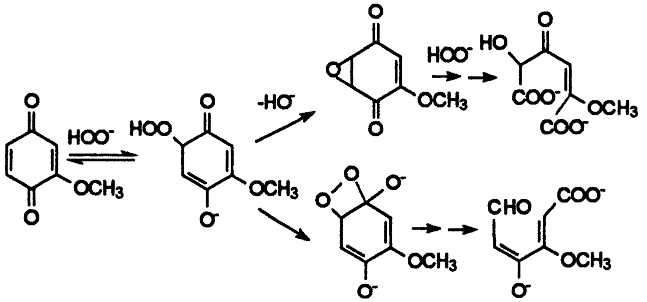
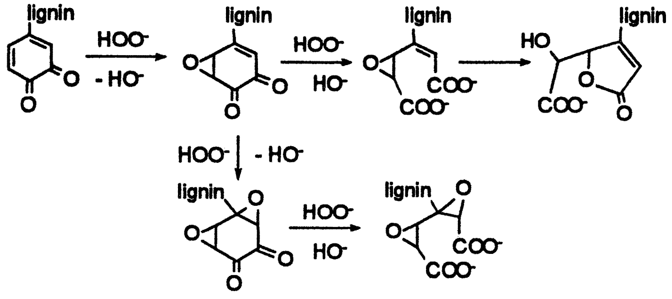


In the hydrogen peroxide oxidation of *o*-quinoid-type structures (shown below) the mechanism involves epoxidation of one or both of the double bonds followed by cleavage between the carbonyl groups (*129*). The resulting mono-epoxide dicarboxylic acids are subsequently converted by intramolecular nucleophilic attack, to the corresponding lactonic acids. Although the reaction with conjugated enone structures is quite facile, it has been observed that hydroxylated quinones, are more resistant to oxidation by hydrogen peroxide than the corresponding non-hydroxylated analogues, requiring higher temperatures and longer reaction times.



Even at temperatures as high as 90°C, alkaline hydrogen peroxide is not reactive towards most nonphenolic lignin structures (*122*). This is due to the fact that hydrogen peroxide is a very weak electrophile and reacts mainly as a nucleophile under alkaline conditions. One way to improve the electrophilicity of hydrogen peroxide is to react it under acidic conditions. Acidic hydrogen peroxide has been used as a pretreatment to enhance delignification in subsequent oxygen stages (*134,135*). Gierer has proposed that under acidic conditions, hydrogen peroxide would be protonated, making it a strong electrophile, leading to direct hydroxylation of the aromatic ring (*136*). However, Kishimoto *et al.* have shown that in the reaction with creosol no hydroxylated products were found, in fact creosol was almost completely

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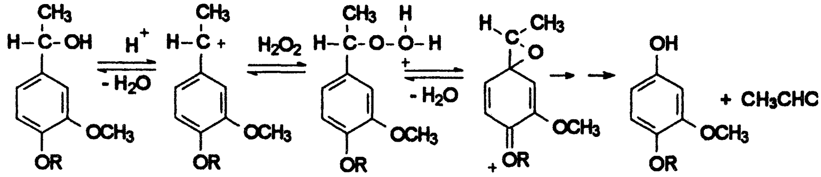
unreacted (*137*). However in the reactions with apocynol and methyl vanillyl alcohol, side chain replacement products similar to those obtained in the Dakin-like reaction in alkaline media were obtained. The reaction appears to go through the formation of a benzylic carbocation followed by nucleophilic addition of hydrogen peroxide. Subsequent protonation facilitates a Dakin-like reaction mechanism, resulting in side chain displacement. Interestingly, both free and etherified phenolic compounds reacted under these acidic conditions.



Unfortunately, problems associated with the practical applications of hydrogen peroxide in acidic media exist. First, a substantial amount of lignin condensation products occur, suggesting that under acidic conditions lignin will condense with its degradation products. Second, hydrogen peroxide decomposes rapidly under acidic conditions with even a trace amount of transition metal ions. Therefore, unless transition metal ions can be completely removed from the pulp, acidic hydrogen peroxide treatments will result in significant deterioration of pulp strength (*138*). Thus the most effective way to increase the electrophilicity of hydrogen peroxide is through the conversion to a peroxy acid.

Reactions of peroxy acids with lignin model systems

Peroxy acids react primarily as electrophiles oxidizing olefinic structures to epoxides. Owing to their electrophilic nature, peroxy acids, in particular peracetic acid (CH_3CO_3H) (*29,112,139-143*), peroxymonosulfuric acid (H_2SO_5) (*29,144-147*), performic acid (HCO_3H) (*148,149*), peroxymonophosphoric acid (H_3PO_5) (*150,151*) and peroxyimidic acid ($NH_2C(NH)O_2H$) (*24,25,30,31,152*) have been extensively studied with regard to their reactions toward lignin model systems (*4,5,27,28,29,135,153*).

The reactions involved include hydroxylation of the aromatic ring, ring opening via *o*-and *p*-quinone formation, side-chain oxidation/elimination and Baeyer-Villiger reactions. Unlike hydrogen peroxide, peroxy acids have been shown to react with etherified phenolic lignin model compounds, although their rate of oxidation is much slower than that observed for their free-phenolic counterparts (*144,154*).

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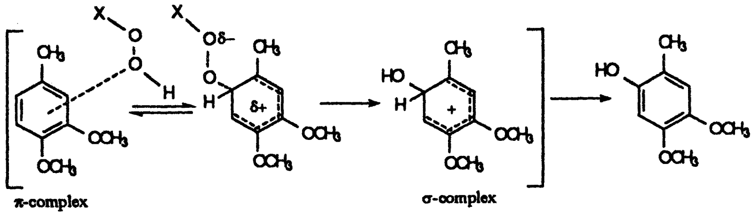
In general, the initial reactions of peroxy acids with lignin can be classified into three main types:

- Electrophilic substitution resulting in ring hydroxylation,
- Nucleophilic addition to a benzylic carbocation followed by side chain displacement, and
- Nucleophilic addition to an α-carbonyl followed by Baeyer Villiger Rearrangement.

1. Electrophilic aromatic substitution

Aromatic hydroxylation is the dominant reaction for lignin structures with saturated side-chains. The reactions are initiated by electrophilic hydroxylation *ortho* and/or *para* to an oxygen bearing substituent (*143*). The reaction involves the formation of a π-complex followed by a rate-determining σ-complex formation (*155*). The rate-determining step is affected mainly by the ability to stabilize the developing positive charge leading to the formation of the σ-complex. Consequently, the rate of the reaction is mostly affected by the leaving ability of the corresponding acid anion (XO$^-$) (*72*).



These initial hydroxylated products, now more reactive towards the peroxy acids, are further oxidized to the corresponding quinoids and aromatic ring cleavage products (*32,143,145*). The initial hydroxylation results in either demethoxylation to the *o*-benzoquinone followed by rapid oxidation to a muconic acid derivative, (top pathway) or hydroxylation and subsequent oxidation/demethoxylation to a methoxy-*p*-benzoquinone or a hydroxy-*o*-benzoquinone (lower pathway). The latter under going further oxidation to the hydroxy- muconic acid derivative and the corresponding lactones.

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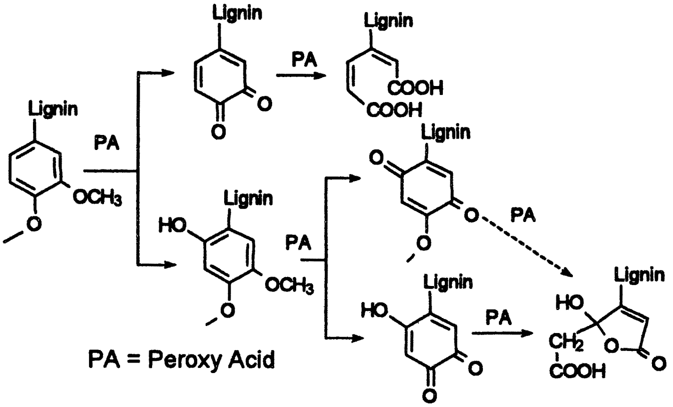
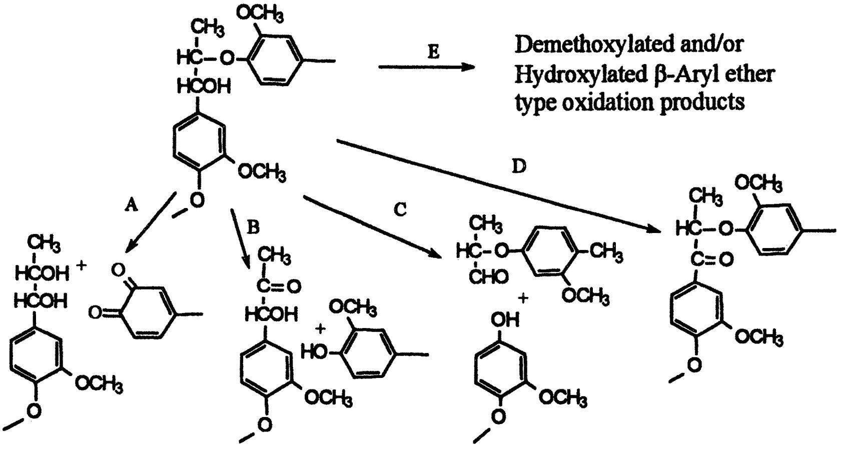

Lignin
PA
OCH3
O
HO
COOH
CH2
PA = Peroxy Acid

In addition to hydroxylation/ring-opening type reactions involving the aromatic ring, direct cleavage of inter-aryl linkages, particularly in β-aryl ether compounds have also been observed (*156*). Three major routes are believed to be involved, one involves side-chain displacement (C), while the other two (A, B) are due to cleavage of the β-aryl ether bond.

Cleavage of the β-Aryl ether bond can be attributed to: (A) oxidative cleavage through the hydroxylation of the β-aryl ether ring *ipso*, or *ortho* to, the inter aryl linkage followed by either reaction with water, or neighboring group participation and oxirane formation. The involvement of a carbonium ion intermediate has been proposed (*157*), however the observed retention of stereochemistry would not be satisfied by such a mechanisms, or (B) hydroxylation of the β-aryl ether ring *ipso* to the inter aryl linkage followed by oxidative hydrolysis and re-aromatization to the corresponding products.


E
Demethoxylated and/or Hydroxylated β-Aryl ether type oxidation products
D
C
A
B
CH3
HC–O
HCOH
C=O
CHO
OCH3
OH
HO
O
+

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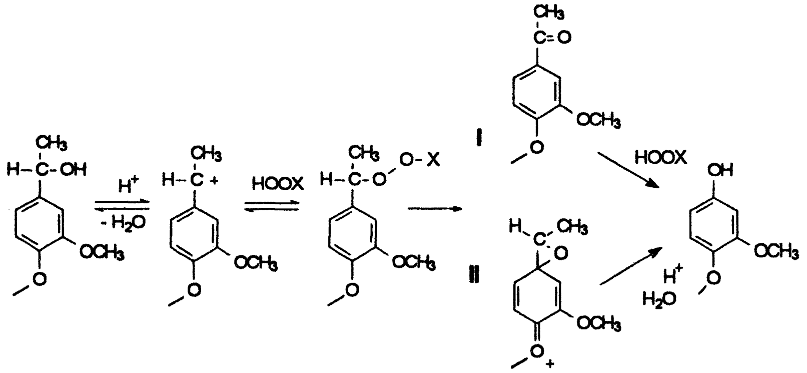
In the reactions of type (C), *ipso* hydroxylation of the non-aryl ether ring is followed by oxidation of the α-hydroxyl group and subsequent degradation of the aryl side-chain (*32,136*). However based on the recent work of Kishimoto *et al.* (*137*) and Zhu *et al.* (*151*) the possible involvement of a benzylic carbocation followed by nucleophilic addition of the peroxy acid and subsequent Baeyer Villiger rearrangement seems most likely. Finally, numerous investigators have also reported the oxidation of the α-hydroxyl group to the corresponding keto compound (D). Although the mechanism is not known, it is thought to involve a free radical process (*29,144*).

2. *Nucleophilic addition to a benzylic carbocation*

Peroxy acids are good nucleophiles and as such, add readily to carbocations or quinone methides. Under acidic conditons, carbocations can be formed from either α-carbinol or α-ethers of both phenolic and non-phenolic units, though the α-ether requires much lower pH (*158*). Nucleophilic addition of the peroxy acid gives a peroxy intermedate, which may rearrange via two different pathways, both resulting in side-chain displacement (discussed above). In pathway I rearrangement gives the α-carbonyl product, which is further degraded through the Baeyer-Villiger oxidation (*vide infra*). In pathway II, the side-chain is displaced through epoxide formation, which is subsequently hydrolyzed via an unstable hemiacetal intermediate, analogous to the Dakin-Like reaction under alkaline conditions.



The transition state again involves the development of a positive charge and it is likely that the leaving ability of the peroxy acid is the main factor determining the rate (*72*). Thus, the rate of the reactions among the various peroxy acids follows the same order as the electrophilic substitution reactions.

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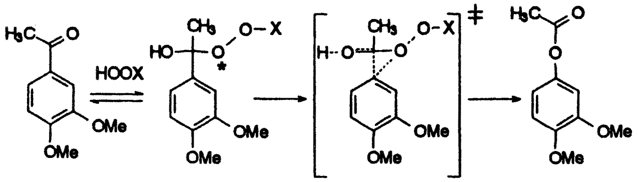
3. Baeyer Villiger rearrangement

The reaction of ketones with peroxy acids is well known to go through the Baeyer Villiger rearrangement (*159*). The initial step is nucleophilic addition of the peroxy acid to the ketone followed by a rate-determining rearrangement to the peroxyester intermediate via a concerted aryl migration to form the acetate ester. The transition state of the rate-determining step likely involves neither the development of carbocation nor the tentative loss of the aromatic structure as in the other two reactions described above. Thus the rate-determining step is influenced conceivably more by the electrophilicity than the leaving ability of the peroxy acid adduct (*72*).



Recently, Chang and coworkers investigated the kinetics of several peroxy acids with various lignin model compounds (*72*). They propose that two main factors affect the reactivity of peroxy acids (XOO*H): 1) the leaving ability of XO^- and 2) the electrophilicity of the peroxide oxygen O^*. The leaving ability of the peroxy acids is estimated by the pKa values for the conjugated acids, XOH, whereby the relative rank of the three peroxy acids they studied were $HSO_5^- \geq H_3PO_5 > CH_3CO_3H > H_2PO_5^- \gg HPO_5^{=}$. The electophilicity of the peroxide oxygen O^*, or the ability of the peroxide oxygen bond to become polarized and form a partial positive charge on the peroxide oxygen O^* is dependent on the inductive effect of the XO group. Since O in the XO group is common to all peroxy acids, the inductive effect of the X group is then responsible for the electrophilicity of the various peroxy acids. The relative rank of the electrophilicity of the peroxy acids investigated was $H_3PO_5 > CH_3CO_3H > HSO_5^- > H_2PO_5^- \gg HPO_5^{=}$.

Finally, peroxyimidic acid, which is generated in the activation of hydrogen peroxide by cyanamide (NH_2CN), has been reported to be isoelectronic with peroxyacids in the bleaching of chemical pulps (*24,25,160*). However, the peroxyimidic acid intermediate has not been isolated nor has there been any evidence that it is the only reactive species formed in this reaction system. Recently, utilizing electron paramagnetic resonance (EPR) spectroscopy and spin trapping, Kadla *et al.* have shown free radical involvement in the cyanamide-activated hydrogen peroxide reaction system

(*29,30,31*). In contrast to previous literature, a predominately free-radical mechanism exists. On the basis of the reactions carried out with superoxide dismutase (SOD), the presence of superoxide ($\cdot O_2^-$) has been determined.

Peroxide decomposition products: hydroxyl and superoxide radicals

As discussed above, peroxides are susceptible to thermal and transition metal induced homolytic fragmentation reactions in which hydroxyl ($HO^\cdot$) and superoxide ($O_2^{\cdot-}$) radicals are generated. The hydroxyl radical ($HO\cdot$) is the strongest one-electron oxidant that can exist in aqueous conditions (*161,162*). As a short-lived but extremely powerful oxidizing agent, the hydroxyl radical is capable of oxidizing a variety of organic and inorganic compounds. Generally, the reaction proceeds by hydrogen abstraction (*78-80,163*), the driving force being the difference between the free energy of bond formation ($-\Delta G_{BF}$), and the enthalpy of bond dissociation for R-H (ΔH_{DBE}). In general most molecules react exothermally with hydroxyl radical, generating a new free radical and water. The newly generated organic radical then rapidly reacts with molecular oxygen to yield the corresponding peroxyl radical, as outlined below.

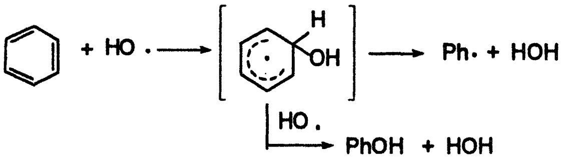
$$HO\cdot + RH \longrightarrow R\cdot + H_2O \qquad (10)$$

$$R\cdot + O_2 \longrightarrow RO_2\cdot \longrightarrow\!\!\!\!\rightarrow \qquad (11)$$

$$HO\cdot + RX \longrightarrow RX^{\dot{+}} + HO^- \qquad (12)$$

These reactive intermediates initiate the chain reactions of oxidative degradation, leading ultimately to carbon dioxide and water.

Although H-atom abstraction is the common path for most substrates, electron transfer constitutes another mechanism of oxidative degradation (*48*). The large bond dissociation energy of aromatic C-H bonds precludes direct H-atom abstraction, but addition to the conjugated π-electron system is energetically favorable, the result being hydroxylation and thus further activation of the aromatic substrate.



From the above reaction schemes it is clear that the rate and efficacy of the oxidative degradation process depends on the bond energy of the substrate and

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the reactivity of the radical intermediates. Thus in the bleaching of wood pulps, which are made up of aromatic (lignin) and carbohydrate (cellulose and hemicellulose) components, the generation of hydroxyl radicals leads to the degradation of both substrates. In fact, studies into the reactions of hydroxyl radicals generated by γ-irradiation, UV photolysis or Fenton's chemistry have shown just this (*163-168*).

Superoxide anion radical ($\cdot O_2^-$) also plays an important role in free-radical reactions and has been extensively studied and documented (*169*). It is now clear that the superoxide displays four basic types of reactions depending on the reaction conditions, deprotonation, H-atom extraction, nucleophilic attack and electron transfer. The majority of research into the behavior of superoxide has been carried out in aprotic solvents. This has been necessary due to the fact that in the presence of a proton or protic source, superoxide rapidly disproportionates to molecular oxygen and perhydroxyl anion (*78-80*).

$$2\,\cdot O_2^- + H_2O \rightleftharpoons HO_2^- + O_2 + HO^- \qquad K \sim 9.1 \times 10^8 \qquad (13)$$

In fact in aqueous media, superoxide functions primarily as a strong Brønsted base, deprotonating weakly acidic organic compounds. Although the equilibrium favors molecular oxygen formation, the rate of disproportionation is pH dependent, having a maximum rate at a pH equal to the pKa of superoxide (~4.5 @ 25°C) and decreasing with increases in pH. As a result, only those reactions that can compete with the rapid disproportionation will be observed in protic media. Superoxide has been reported to undergo hydrogen-abstraction reactions (*31,170*), however, as with molecular oxygen, substrate activation is required, i.e. heat, ionization, etc. Kadla *et al.* have demonstrated that even at 90°C in alkaline conditions, nonphenolic lignin structures are unreactive towards superoxide (*30,31*).

Numerous researchers have also proposed a radical-radical coupling mechanism for superoxide with other generated radicals (*171,172*) to yield the corresponding hydroperoxide anion, and the accompanying ring-opening reactions. Although this is quite typical of free-radicals, it is not necessarily common for radical anions (*173*). There are also several documented cases where superoxide does not react with free-radicals by coupling, but rather reduces them (*174-176*). Due to the complex nature of superoxide chemistry, it is important to note that a variety of autoxidation reactions can take over after the initial superoxide reaction; therefore caution must be taken when attempting to analyze the mechanism of superoxide by simply examining the generated products.

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Chapter 7

A Detailed Study of the Alkaline Oxidative Degradation of a Residual Kraft Lignin Model Compound

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The kinetics of alkaline peroxide oxidative degradation of a residual kraft lignin model compound, namely 3,3'-dimethoxy-5,5'-dimethyl-[1,1'-biphenyl]-2,2'-diol (I) were examined. Activation energies for both the consumption of I and the evolution of the main degradation product, 2-hydroxy-3-methoxy-5-methylbenzoic acid were not significantly different (117±2 and 116±3 kJ/mol, respectively). Computational analysis supports the notion that the degradation is driven primarily by reactions with hydroxyl radicals, leading to the formation of radical sites. Subsequent radical couplings yield organic peroxides and then dioxetane structures. Formation of a dioxetane intermediate is a prerequisite for the final fragmentation of the molecule and computations suggest that this is the rate-limiting step in the oxidative degradation pathway of I. A transition structure was computed whose formation requires energy input (+132 kJ/mol) similar to the experimentally determined activation energy.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
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Introduction

The alkaline oxidative degradation of lignin and lignin model compounds is a complex chemical process whose fundamentals have been examined in a number of studies (1,2,3,4,5,6,7,8). The process can be described as a series of reactions between the substrate, oxygen, and hydrogen peroxide in the presence of alkali and traces of transition metals. This combination of reactants is known to result in the formation of hydroxyl and hydroperoxyl radicals, superoxide radical anions, as well as non-radical hydroxide and hydroperoxide anions. The large number of intermediates that are formed in the process (9) suggests that the degradation of aromatic substrates follows a variety of pathways. These pathways include demethylation reactions, oxidative ring cleavage, oxidative side-chain scission, various lactonization reactions, radical coupling reactions, epoxide formation, decarboxylation reactions, carbonyl group formation, etc. The primary reactions mainly involve electrophilic hydroxyl and peroxy radicals. Nucleophiles such as hydroperoxide and superoxide radical anions ($\bullet O_2^-$) also play a vital role in these reactions.

Industrial interest in the alkaline oxidative degradation of lignin stems from an effort to improve the efficiency and minimize the environmental impact of pulp bleaching. The use of alkaline hydrogen peroxide may contribute toward this goal. A significant number of research endeavors have focused on improving the efficiency and the selectivity of the alkaline hydrogen peroxide bleaching stage. Augmenting its efficiency relates to reducing the amount of costly hydrogen peroxide required, while improving the selectivity of the process, i.e. focusing its reactivity toward lignin, which would result in stronger pulp and higher process yields. Detailed knowledge of the reaction pathways occurring during the interaction of alkaline hydrogen peroxide with lignin moieties could allow for beneficial process modifications.

In relation to the reactivity of pure alkaline hydrogen peroxide, Gierer pointed out that this oxidant does not attack phenolic rings (10). Its action is confined to nucleophilic addition reactions on carbonylic centres. Gierer, Yang, and Reitberger (9) have proposed in their study with radiolytically generated hydroxyl radicals, that radical reactions could be the principal species responsible for the oxidative degradation of lignin. There are several possible reactions of hydroxyl radicals with lignin (10), including those that lead to the formation of radical sites on lignin. These sites can then engage in coupling reactions with superoxide radical anions. Hydroxyl and superoxide radicals are formed from the interaction of hydrogen peroxide with traces of transition

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metals. These radical addition reactions eventually lead to oxidative ring opening and α-β scission causing the eventual extensive fragmentation within lignin.

A new milestone in lignin chemistry was reached when Brunow's group announced the discovery of dibenzodioxocins as being a substantial integral part of the lignin building blocks (11). This discovery was then coupled with studies of dibenzodioxocin degradation under alkaline and kraft pulping conditions showing that dibenzodioxocins cause the formation of alkylated 5,5'-bicreosol type moieties within residual kraft lignin (12). Ahvazi et al. (13) have recently shown that milled wood lignin subjected to kraft pulping conditions liberated large amounts of stable 5,5'-bicreosol moieties, which eventually became an integral part of the residual kraft lignin structure. The significance of such moieties in understanding the industrial oxidative degradation of kraft pulp becomes apparent.

For the case of residual kraft lignin, 3,3'-dimethoxy-5,5'-dimethyl-[1,1'-biphenyl]-2,2'-diol (I) could be considered as one representative model structure (14). This compound has also been termed (6,6')-bicreosol, or (5,5')-bicreosol, and early investigations of its alkaline oxidative degradation were done in the sixties (1).

In our latest published account aimed at further comprehending the interaction of lignin-like moieties under oxidative conditions we reported, amongst others, the detailed reaction products of the interaction of alkaline H_2O_2 with I, (15). Our data showed that this reaction is accelerated by the presence of transition metals, in accordance with Gierer's (10) notion of the radical nature of these reactions. 2-Hydroxy-3-methoxy-5-methylbenzoic acid and 2-hydroxy-3-methoxy-5-methylbenzaldehyde were among the degradation products detected and quantified, suggesting that the alkaline peroxide system behaves in a manner similar to that of alkaline-oxygen described earlier by Kratzl *et al.* (1).

The present effort attempts to delve further into the oxidative degradation of I with alkaline H_2O_2 by building upon our earlier data. Measurements of the activation energy of the reaction together with the detailed reaction product profiles, determined earlier (15), were coupled with appropriate computations aimed at formulating a detailed degradation pathway. These studies permitted the determination of the reactive intermediates that most likely control the rate-determining steps in these reactions.

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Experimental

Materials

Nano pure deionized distilled water was used to prepare all solutions. $MgSO_4$ (99.99 + %), NaOH pellets (99.99%), 3,4-dimethoxyphenol (97%), and KI (99.9%,) were purchased from Aldrich Chemical Company, Inc. H_2SO_4 (95.0 ~ 98.0%) and ethyl acetate were from Caledon Inc. HCl (36.5~38.0%) was from Fisher Scientific Co. The model compound (I) was synthesized according to the procedure reported by Douglas et al. (16).

Alkaline H_2O_2Treatment

3,3'-Dimethoxy-5,5'-dimethyl-[1,1'-biphenyl]-2,2'-diol (I), was treated with H_2O_2 under alkaline conditions at 100°C for 20, 40, 60, and 120 minutes; at 110°C for 10, 20, 30, and 60 minutes; and at 120°C for 10, 20, 30, and 60 minutes using a 40 mL stainless steel reactor. To minimize metal ion contamination, a customized glass liner with a glass cover was used within the reactor. The molar ratio of compound I to H_2O_2 and to NaOH was 1:6:4. I, (100 mg) was accurately weighed and placed in the glass liner. Deionized distilled water (40 mL), $MgSO_4$ (1.23 mg) and NaOH (58.5 mg) were consecutively added to the model compound. Dissolution was effected by stirring with a magnetic stirrer for 10 minutes. The pH before the addition of H_2O_2 was around 12. Finally, 0.20 mL of H_2O_2 solution (37%) was added. The glass liner was immediately covered with the glass cover and inserted into the reactor which was then placed in a preheated oil bath. When the desired reaction time was reached, the reactor was rapidly cooled with running water for 5 minutes. Two mL of the reaction mixture were then withdrawn for titrating the amount of residual H_2O_2. Depending on the rection conditions, the residual H_2O_2 was in the range of 1.2-98 % of the original. The pH of the remaining solution (8.4-12.0) was then adjusted to about 2 with 1 N HCl. The acidified mixture was freeze-dried and the resulting solid was extracted four times with 10 mL aliquots of ethyl acetate. The extract was finally vacuum dried and analyzed by GC/MS.

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GC/MS Analyses

GC/MS analyses were carried out with a Hewlett Packard 5982 mass spectrometer interfaced with a Hewlett Packard 5890-A gas chromatograph equipped with DB-5 x 0.25 packed silica capillary column. The temperatures for the injection port and the detector were 250 and 280°C respectively. The oven temperature was raised from 70°C to 250°C with a gradient of 15°C/min. The products were silylated using N,O-bis(trimethylsilyl)acetamide before GC/MS analysis and then identified by comparison with the MS library or with spectra of authentic samples.

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Product Quantification

The quantification of the products was carried out by Gas Chromatography (GC) coupled to a Flame Ionization Detector (FID) using the method specified for the GC/MS analysis above. 3,4-Dimethoxyphenol was used as a GC internal standard. Only two compounds were quantified, namely, the remaining compound I and the main product of the oxidation, 2-hydroxy-3-methoxy-5-methylbenzoic acid (compound XIII). The GC response factors of these two compounds relative to the standard were determined by mixing the internal standard and the corresponding compound, followed by GC area measurements.

Computational Methods

The initial structures of all compounds depicted in Schemes 1-5 were produced using a model builder (HyperChem 5.11 Professional, HyperCube, Inc.). The structures were subjected to geometry optimization with the MM+ molecular mechanics (17) force field (to root mean square, RMS, gradient kJ·mol^{-1}Å^{-1}). A single-point semi-empirical PM3 (3rd parametrization method; 18,19) computation was then performed to assign partial charges on all atoms. The charges permitted to use an improved MM+ force field, based on non-bonded electrostatic interactions. Using this force-field, we generated the starting structures by one of the following methods. For structures with two rings or long side chains we searched for energy minima by systematic incremental rotations around the main bonds. For simpler structures we used simulated annealing. The starting geometries were then minimized with MM+ and finally with the PM3 semi-empirical method (18,19) with self-consistent-field convergence set to 0.01 and the optimization convergence to RMS gradient 0.2 $kJ{\cdot}mol^{-1}Å^{-1}$. The unrestricted Hartree-Fock (UHF) algorithm was used. The atomic charges, atomic orbital electron populations, and heats of formation were computed from these energy-minimized structures.

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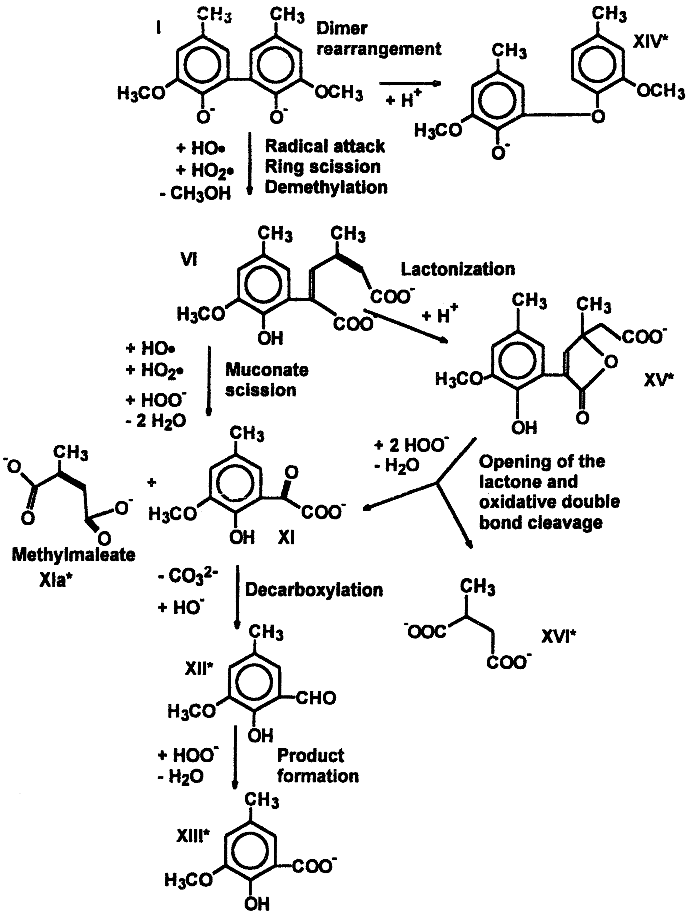
Results and Discussion

Kinetic Studies

The reaction between I and H_2O_2 was carried out under alkaline conditions. Under the most extreme conditions (120°C for 60 min), the pH dropped to about 8.5 after the reaction. The molar ratio of 6/1 for H_2O_2/I used in this experiment was enough to ensure the presence of H_2O_2 under all of the conditions of the kinetic study (at least 1.2 % of the original H_2O_2).

The activation energies for the oxidative elimination of I and the formation of 2-hydroxy-3-methoxy-5-methylbenzoic acid (XIII) were calculated from the kinetic data using the Arrhenius plot. The activation energy for the disappearance of I was determined to be 117±2 kJ/mol while that for the formation of XIII was 116±3 kJ/mol. Thus both activation energies were the same, within the experimental error, suggesting the presence of a single rate-determining step in the oxidative degradation pathway of I. The energy barrier responsible for the activation energy is due to an energy input required to move the degradation reaction from a certain intermediate to a transition state. Knowledge on the precise nature of this barrier could eventually lead to improved reaction conditions or assist the design of a catalyst aimed to decrease this energy barrier. These improvements could eventually permit faster reaction rates or milder reaction conditions. Improvements in either direction will be highly desirable from the technological standpoint. In an effort to gain additional insight about the reaction's details and its rate determining steps, we employed computational analysis of the degradation pathway as described below.

The Degradation Pathway


I
Dimer rearrangement
+ H+
XIV*
+ HO•
+ HO2•
- CH3OH
Radical attack
Ring scission
Demethylation
VI
Lactonization
+ H+
XV*
+ HO•
+ HO2•
+ HOO-
- 2 H2O
Muconate scission
+ 2 HOO-
- H2O
Opening of the lactone and oxidative double bond cleavage
Methylmaleate
XIa*
XI
- CO32-
+ HO-
Decarboxylation
XVI*
XII*
+ HOO-
- H2O
Product formation
XIII*

Scheme 1 An overall scheme of compound I degradation. The asterisks denote compounds that were identified experimentally by Sun et al. (15).

In this section our efforts in delineating the mechanism for the oxidative degradation of I will be described. Its foundations reside with the experimentally identified degradation products reported by Sun *et al.*

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(15), data obtained from the computational analysis of the components of the pathway, and literature accounts published by the groups of Kratzl, Gratzl, Dence, Gierer, Reitberger, Kadla, and others (see References).

A simplified pathway accounting for all the degradation products identified by Sun *et al.* (15) is shown in Scheme 1. The main direction of this pathway, includes compounds I, VI, XI, XII, and the principal degradation product, XIII. The latter three compounds were among those determined experimentally by Sun *et al.* (15). Since compound XIV was also detected by Sun *et al.*, its formation could tentatively be accounted via a rearrangement of I. The presence of XV among the products can be interpreted as a consequence of lactonization of VI, in analogy with the pathway for the alkaline autooxidation of 2,4-dimethyl-6-methoxyphenol proposed by Gierer and Imsgard (20,21). Subsequent opening of the lactone and the oxidative cleavage of the double bond could lead to the formation of another product identified by Sun et al., 2-methylsuccinate (XVI), and XI.

The main pathway of the Scheme 1 (compounds I,VI,XI-XIII) was then expanded to include a complete set of intermediates and its detailed course is shown in Schemes 2-5. This complete main pathway was subjected to an extensive computational analysis. The feasibility of the existence of the various intermediates, proposed in Schemes 2-5, was supported by computational analysis methods aimed at characterising how the predicted properties of a specific structure fit within the proposed sequence of chemical reactions. Often the previous art suggested a number of options for the steps along the pathway (e.g. various ionization states, types of oxygen containing rings, and radical sites). In such cases we examined the options computationally and chose those thermodynamically most favourable. In this way the computations provided valuable feedback during the process of building the pathway and several modifications were made by an iterative process. This is not to suggest that the proposed pathway is the only one, but rather that amongst the many possible pathways this one is the most probable.

The chemistry described in Schemes 1-5 assumes (and the assumptions are supported by the computations) that under the alkaline conditions of these reactions all carboxylic acid groups are negatively charged, while all hydroperoxyl and aliphatic hydroxyl groups are protonated. As for the

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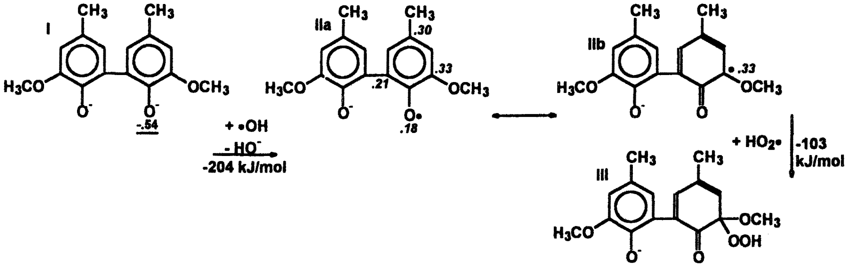
phenolic hydroxyl groups an assumption was made that these are dissociated unless there is a carboxylate group in the molecule. The carboxylate probably causes an increase of the pK_a of the phenolic hydroxyl.

In accordance with the accounts of Gierer (10) we thought that hydroxyl radicals generated from H_2O_2 represent the main driving force in this degradation. The radicals abstract hydrogen atoms from the substrate and produce radical sites. These sites can further react with superoxide radical anion, $\bullet O_2^-$ or its conjugate acid, hydroperoxyl radical, $\bullet O_2H$. The radical coupling reactions introduce hydroperoxyl groups onto some of the intermediates of the pathway and eventually lead to C-C bond scissions. These scission reactions are of paramount importance to the degradation process of residual lignin since they initiate its breakdown into smaller fragments. They also allow the introduction of carboxyl groups on the substrate which consequently increase its solubility in alkali.

The computations discussed below support the notion of a cooperative action between hydroxyl and hydroperoxyl radicals with the substrate. The oxidative degradation of I (Scheme 2) commences with the formation of its dianion. Compound I is strongly electronegative at the phenolate oxygens (charge -0.54). Therefore, either of these oxygens is a likely site for a reaction with the electrophilic hydroxyl radical. The



Scheme 2 The initial radical reactions leading to the formation of a peroxide. The underlined numbers denote computed partial charges; the numbers in italics are computed spin populations.

ensuing peroxide radical intermediate releases hydroxyl anion. A phenoxy radical, IIa, is thus formed. The phenoxy radical, IIa, has the highest spin population (0.33) on the carbon to which the methoxyl is bonded. The calculated spin population densities shown in Scheme 2 are

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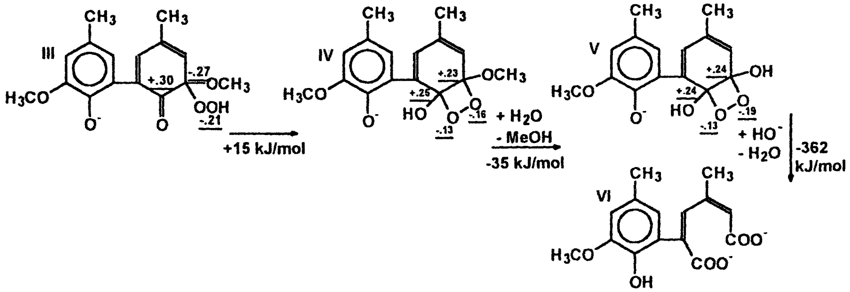
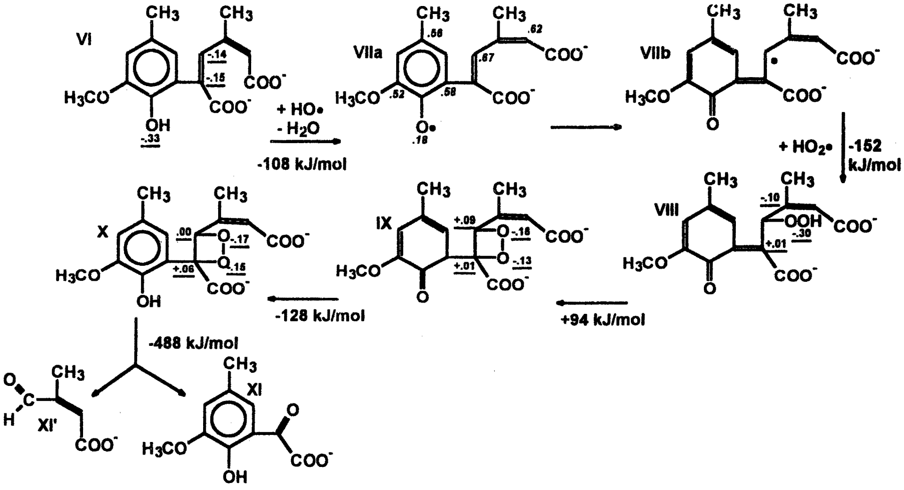
in general agreement with those computed for the coniferyl alcohol phenoxy radical by Martensson and Karlsson (22) and more recently by Elder and Worley (23). This lends support to the resonant structure, IIb, in Scheme 2. Kratzl *et al.* (1) have actually suggested the formation of such a radical. The high spin population density favours an addition of a hydroperoxyl radical leading to the formation of the organic peroxide, III. The formation of a similar peroxide during the alkaline oxidative degradation of I has been suggested by Kratzl *et al.* (1). In this radical coupling reaction (as well as in subsequent ones shown in Scheme 4) we are assuming that hydroperoxyl radical rather than superoxide radical anion is the active species in spite of its low pK_a (4.88). We have considered superoxide radical anion in our computations also but in all cases the results indicated unreasonable endothermic reactions. Under the conditions of these experiments hydrogen peroxide is assumed to be present mostly as the hydroperoxide anion, HOO^-. It has been pointed out by Kadla *et al.* (24*; in reference to Sawyer,*25) that the perhydryl radical (the conjugate acid of superoxide radical anion) may be present even under alkaline conditions despite its low pKa (4.88). This will be due to the rapid disproportionation of $\bullet O_2^-$.

Compound III contains a hydroperoxyl group with a negative charge of -0.21 on the terminal oxygen (see Scheme 3). The nucleophilic



Scheme 3 Formation of dioxetane, demethylation and ring cleavage. The underlined numbers denote partial atomic charges.

hydroperoxyl group is thus likely to react with the most elecropositive atom in the vicinity (C with charge +0.30). This results in the formation of a dioxetane ring in compound IV. The dioxetane intermediate IV contains a ring with bond angles on the oxygen atoms 90° and 91°, compared to 116° for the methoxyl group oxygen. Both dioxetane carbons are electropositive (+0.25 and +0.23 resp.) and the corresponing dioxetane oxygens are electronegative (-0.13 and -0.16. resp.). Due to electrostatic repulsions and the strained bond angles the structure is high in energy and an endothermic reaction is required for its formation. In actual fact, Gierer (10) proposes ring cleavage of a similar dioxetane structure before demethylation for the same reacton. Our calculations, however, indicate that demethylation of V preceding its ring cleavage is energetically more favourable. Structure V which is formed via demethylation remains an unstable moiety due to the presence of the dioxetane ring. Consequently, the structure V undergoes ring cleavage with a considerable release of energy. A dicarboxylic acid, a muconate derivative, VI, is thus formed.



Scheme 4 Radical reactions leading to scission of the muconate derivative. Spin densities are in italics; partial charges are underlined.

The most likely site on VI for electrophilic attack by a hydroxyl radical is the hydroxyl oxygen with negative charge -0.33 (see Scheme 4). Hydrogen abstraction yields a radical with the highest spin population

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(0.67) on the aliphatic side chain. This makes it a preferred site for an attack by the hydroperoxyl radical with the subsequent formation of an organic peroxide, VIII. In a reaction analogous to the one shown in Scheme 3, a dioxetane ring is formed in IX by an endothermic reaction. The dioxetane then rearranges exothermically to form an aromatic compound, X, which in the next step fragments by a highly exothermic dioxetane scission to yield two carbonyl structures: an α-keto acid, XI, and methylmaleyl aldehyde, XI'. A reaction pathway leading to a similar scission reaction has been postulated by Kratzl et al. (1) for the alkaline oxidative degradation of I involving an oxirane intermediate. We examined this option computationally and found it less favourable. Threfore in this effort we assume that the scission is analogous to a conjugated double bond cleavage that occurs via a dioxetane intermediate as discussed earlier by Gierer (10).

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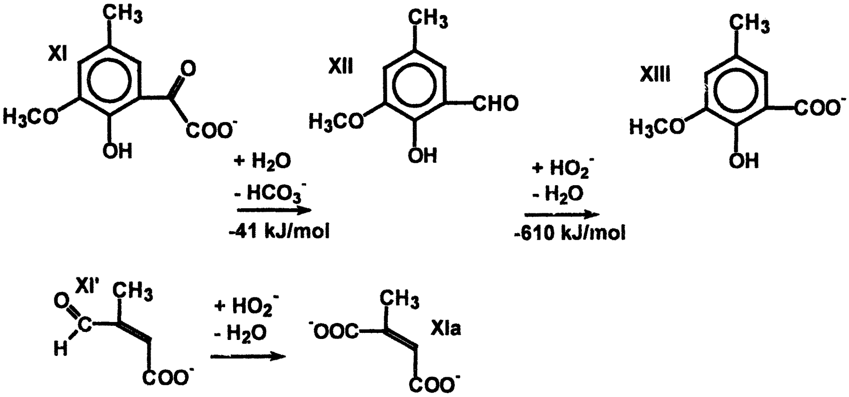

Scheme 5 Final decarboxylation and oxidation reactions of the muconate scission products.

The scission products, XI and XI' undergo further reactions shown in Scheme 5. Hydroperoxde anions that are in excess within the system oxidize the aldehyde, XI', to methylmaleate, XIa. Methylmaleate has actually been detected by Sun *et al.* (15) amongst the degradation products of I with alkaline peroxide. Compound XI is an α-keto acid which readily decaboxylates with the formation of carbonate to yield an aromatic aldehyde, XII, which has also been found among the oxidative degradation products by Sun et. al. (15). Furthermore, XII oxidizes to XIII, 2-hydroxy-3-methoxy-5-methylbenzoate, in the presence of hydroperoxide anion. Compound XIII has been detected as a major degradation product of I by Bailey and Dence (26), Kratzl *et al.* (1) and Sun *et al.* (15).

Reagents and Reaction By-products of the Main Degradation Pathway

In addition to compounds I – XIII, Schemes 1-5 also show a number of low molecular weight compounds all of which were taken into account in the reaction balances. All these compounds participate in the pathway either as reagents (some arising from alkaline hydrogen peroxide) or are formed as by-products of the oxidative degradation of I. Heats of formation (in kJ/mol) for these compounds, computed by the same procedure as for the intermediates, were as follows: $HO\bullet$, +12; HO^-, -73; $HO_2\bullet$, -11; H_2O, -223; CH_3OH , -218; XI', -614; HCO_3^-, -743; HO_2^-, -101. These compounds were used to balance the reactions of the main pathway (Schemes 2-5). Their heats of formation, along with the computed heats of formation of compounds I-XIII, were used to complete the energy balance calculations whose results are plotted in the energy diagram (Figure 1) and discussed below.

Energy Balance of the Main Degradation Pathway

Garver in 1996 (27) demonstrated the usefulness of computational methods for better understanding bleaching reactions. He used the PM3 semi-empirical method to compute the heats of formation for a number of compounds involved in a lignin degradation pathway and compared the results with experimentally determined activation energies. In a similar manner, we explored the thermodynamics for the oxidative degradation of I by computing the heats of formation of the various intermediates using the PM3 energy-minimized structures. To test the PM3 method against the benchmark computed data of Dewar *et al.* (28), we computed the heats of formation of 13 compounds (not shown) using our protocol and compared the results with published data. Ten of the computed values agreed closely (within less than 3 kJ/mol). For the case of diphenylmethane, however, a value lower by 29 kJ/mol to the experimental value was obtained. This was probably due to a different optimized geometry. For the cases of allyl and t-butyl radicals the computed data were also lower by 36 and 14 kJ/mol, respectively. When, however, instead of UHF we employed a faster algorithm using a one-

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electron approximation (restricted Hartree-Fock), an agreement within 12 and 1 kJ/mol respectively was apparent.

It is important to mention at this point that both the benchmark data of Dewar *et al.*(28) and our computations were carried out *in vacuo* without

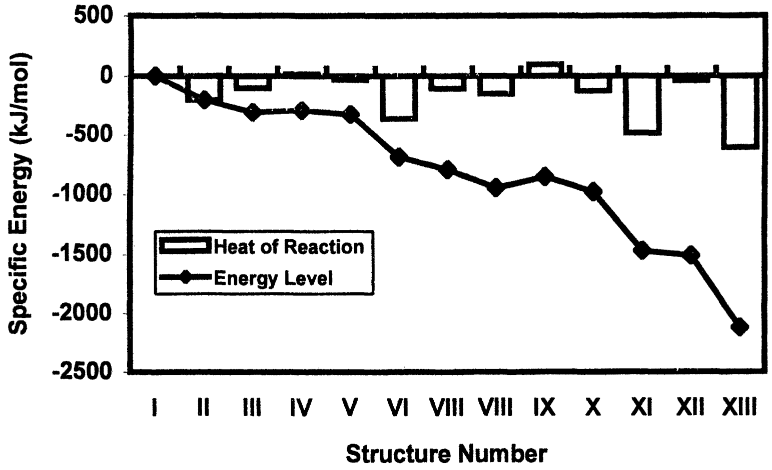

Figure 1 Energy diagram constructed from the computed heats of formation of all compounds in the proposed degradation pathway (Schemes 2-5). The bars show the computed heats of reactions for the individual steps. Positive values for structures IV and XII indicate endothermic reactions. The line represents the generally decreasing energy level along the pathway.

taking into account the presence of the solvent. This omission was due to the computational difficulty of dealing with a more complex system (including a large number of solvent molecules, i.e. water and counter ions). In an effort to clarify if our conclusions could be seriously affected by possible solvation effects we attempted to compute the energies of solvated reaction intermediates using the MM+ force field procedure instead of the more stringent PM3 semi-empirical method, thus reducing the otherwise extensive computation time requirements. The accumulated data (not shown) indicated that the observed trends under solvated conditions were qualitatively similar to those of the *in vacuo* semi-empirical computations. In general, however, the noise was greater, leading in several cases to unrealistically high values of heats of reactions.

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The computed heats of formation of compounds I to XIII and the heats of formation for reagents and by-products listed above were used to calculate energy balances for all the steps of the main pathway. The computed results show that almost all the steps on the proposed pathway are exothermic (Figure 1) with two exceptions. The transitions between structures III and IV and between VIII and IX appear to be endothermic, requiring energy inputs of approximately 15 and 94 kJ/mol, respectively. Interestingly, both of these transitions involve the formation of dioxetane rings from their peroxide precursors, while the subsequent scission reactions, leading to acids VI and XI, respectively, are highly exothermic.

Prediction of the Reaction Barrier and Activation Energy

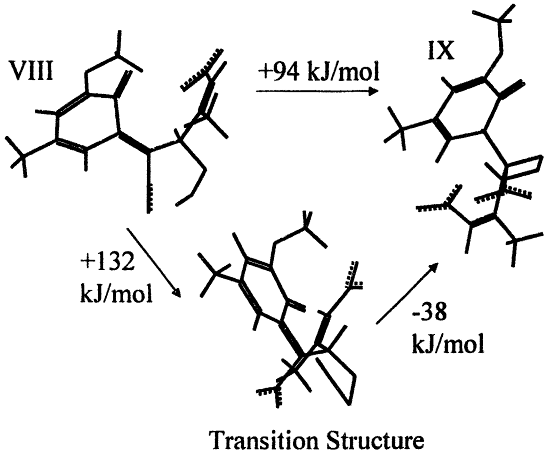

Figure 2 A hypothetical structure providing an intermediate between peroxide, VIII, and dioxetane, IX.

When one attempts to draw conclusions from the computed heats of formation and their relationship to activation energy, the inherent errors of the method have to be born in mind. For the PM3 method the mean unsigned error (average of absolute differences between the computed and experimental values), based on a large number of compounds, was determined by Dewar *et al.* (28) to be 19 kJ/mol for neutral closed shell molecules and 51 for radicals and ions. But, as Dewar *et al.* (28) pointed out, the errors are not random but rather similar for similar compounds. Therefore, computations of differences of heats of formation amongst similar compounds are more reliable than it would appear from the overall error analyses. Even AM1, an earlier semi-empirical method (29), has proved to be useful for computing heats of reaction and activation energies (28). In this respect our approach is fully justified.

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The data of Figure 1 allow one to speculate on the nature of the transition states which are the rate-limiting steps in the degradation pathway. These transition states could be primarily responsible for the experimentally determined activation energies of the degradation reactions of I in the presence of alkaline H_2O_2. Since the activation energy is the energy that has to be provided for a critical intermediate to be transformed into an unstable transition state compound which then spontaneously changes into a low-energy intermediate (from which the reaction is unlikely to proceed backwards), it is likely that for a reaction system such as the one we are examining, the transition states are likely to be found in the endothermic reaction steps. At this point it is logical to speculate that the critical transition state for the oxidative degradation of I is similar to structure IX produced by the most endothermic reaction on the pathway. This reaction, leading to the formation of the second dioxetane ring on the pathway (see Scheme 4), appears to require at least 94 kJ/mol. The only other endothermic reaction on the pathway is that between structures III and IV. This is also a C-C cleavage reaction involving a dioxetane intermediate. Our computations, however, point to lower energy requirements than that between compounds VIII and IX. . Our focus on the transition from peroxide IX to the dioxetane X is also supported by the fact that the transformation requires extensive rearrangement of the molecule, presumably via a highly distorted transition structure. Figure 2 highlights the difference between structures VIII and IX.

The heat of reaction required to produce the intermediate is somewhat lower than the activation energy. This suggests that the transition structure responsible for the activation energy is most likely an intermediate between structures VIII and IX. We have endeavoured to compute the structure of the transition state using a quadratic synchronous transit method with PM3 as implemented by HyperChem. The HyperChem method is based on the transition state search method of

Peng and Schlegel (30). The.transition structure is shown in Fig. 2. The computed energy, +132 kJ/mol, required for transition from VIII to the transition structure roughly agrees with the experimental activation energy, 116-117 kJ/mol.

Conclusions

We have applied an integrated approach to the study of the alkaline oxidative degradation of a residual lignin model compound (I). The approach consisted of analyzing the degradation products, determining the activation energies, proposing a reaction pathway, and subjecting all the reaction intermediates to computational analyses. The results of these analyses and previous published information on the fundamentals of this complex process led us to the following conclusions:

- The alkaline oxidative degradation of condensed lignin structures follows a main pathway that yields organic acids and carbonate as final products.
- The computed properties of the intermediates support the notion that these reactions are of a radical nature, with hydroxyl radicals generating radical sites on lignin and hydroperoxyl radicals adding to the sites.
- The major part of the degradation pathway consists of simple exothermic reactions with the exception of two endothermic steps involving the formation of dioxetane intermediates.
- The first dioxetane ring formation precedes the aromatic ring cleavage, while the second precedes further scission of the opened ring.
- Formation of the second dioxetane ring requires a set of complex transformations in terms of molecular geometry and therefore a substantial energy input.
- A transition state preceding the formation of the second dioxetane intermediate appears to be the rate limiting step in the oxidative degradation pathway of I.

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Chapter 8

Improving Oxygen Delignification with Peroxymonosulphate: Fundamentals

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Peroxymonosulphate (PMS) has been evaluated as a delignifying agent under neutral or acidic conditions. Our findings show that under alkaline conditions, PMS, although unstable, is a very selective and efficient delignifying agent. PMS can be added directly to an oxygen delignification stage. Using a 1% active oxygen charge, a softwood pulp delignification greater than 70% is obtained with good selectivity. This provides higher delignification in the O stage, resulting in increased system closure and reduced chlorine dioxide requirement.

There has been a steady increase in the use of oxygen delignification worldwide since the first commercial installation in South Africa in 1970 *(1)*. The pace of acceptance has increased with the need to decrease the discharge of organic compounds, and particularly chlorinated organic compounds, into the environment *(2)* . These discharges are decreased because of the decrease in lignin content of the pulp entering the bleach plant after oxygen delignification. The degree of delignification achieved in the oxygen stage is ultimately limited by pulp strength considerations but can also be limited by system configurations *(3)*. Recent surveys *(4-5)* have shown that the degree of delignification in mills can vary from 20-60% with an average around 40%. Most mills using oxygen delignification would like to be able to obtain further selective delignification in this stage and continue the process of progressive system closure. One approach is to add recovery-compatible oxygen-containing chemicals to the stage.

Peroxymonosulphuric Acid Bleaching Under Acidic and Neutral Conditions

Peroxymonosulphuric acid (PMS) has been used to increase the delignification prior to the bleaching stages. Since the first patent from Cael *(6)* on the use of PMS in a single stage, many reports have been published on PMS delignification. PMS treatment has been proposed as a single stage *(7-10)*, as a pretreatment including a wash prior to oxygen delignification or ozone *(11-13)*, as an acidic post-treatment after oxygen delignification (with a wash in between) *(14)*, as an interstage (with wash) in the middle of two-stage oxygen delignification *(15-17)*, in combination with ozone *(18)*, or as a final brightening stage *(19)*. Table I shows the various experimental conditions used for the different types of pulp treatments with PMS (the unofficial symbol Px will be used to identify a PMS stage).

In almost all these studies, the Px stage is shown to be quite selective toward lignin. However, a patent by Lam *et al.* *(9)* indicates that the selectivity is about three times greater under neutral conditions than slightly alkaline conditions. In all cases except one *(18)*, PMS has been used in a separate stage with a full wash and in no case has it been used as a single stage combined with oxygen delignification. In most cases the Px stage has been run under acidic conditions which requires long reaction times even at elevated temperatures.

Peroxymonosulphate Decomposition

Peroxymonosulphuric acid ($HOSO_2OOH$) has two ionization constants. The hydroxy proton is strongly acidic ($pKa_1 < 0$) while the perhydroxyl proton is weakly acidic ($pKa_2=9.4$) *(20)*. Initially studied by Ball and Edwards *(21)*, the kinetics of spontaneous PMS decomposition (Equation 1) has been revisited recently *(22-23)*.

$$2\,HSO_5^- \rightarrow 2\,H^+ + 2\,SO_4^{-2} + O_2 \qquad (1)$$

In the pH range from 5 to 9, the spontaneous decomposition of PMS follows second order kinetics and the dependence on the concentration of hydrogen ion is of -0.7 order as represented by Equation 2. The maximum rate of decomposition occurs at a pH corresponding to the pKa_2 (9.4) where the half-life of PMS is less than two minutes *(23)*. This rapid decomposition is the main reason why researchers have been reluctant to study PMS delignification under alkaline conditions.

$$-\frac{d\,[HSO_5^-]}{dt} = k_a \frac{[HSO_5^-]^2}{[H^+]^{0.7}} \qquad (2)$$

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Table I. Conditions for PMS delignification stages proposed in the literature

	pH	*Time*	*Temperature (°C)*	*PMS (%AO)*
Full Stage				
Cael *(6)*	3.0-11.0	30-180 min	40-80	0.05-0.31
Springer *(7)*	< 2	1-12 days	22	0.05-0.69
Seccombe *et al. (8)*	Acidic	210 min	80	0.71
Lam *et al. (9)*	7.0-7.6	30-180 min	20-80	0.094-2.4
Arnold *et al. (10)*	< 5	90-120 min	55-70	0.12-0.25
Prior to O or Z				
Springer *(11)*	5	2-48 h	22	0.45
Meier *et al. (12-13)*	Acidic	15-120 min	25-100	0.05-1.5
After O				
Troughton *et al (14)*	Acidic	70-150 min	75-100	0.5
Interstage OPxO				
Allison *et al. (15)*	3.2-6.3	30 min	75	0.062-0.42
Allison *et al. (16-17)*	7	45 min	75	0.14-0.71
Combined (PxZ)				
Francis *et al. (18)*	4	> 120 min	60	0.47
Brightening				
Sundara *et al. (19)*	Alkaline	2-8 h	45-65	0.05-0.3

NOTE: ***The PMS charges used in the literature are expressed as % of active oxygen (AO) on pulp (One mole of active oxygen per mole of H_2SO_5).***

PMS is quite insensitive to the presence of most transition metal ions but some decomposition occurs with copper under mildly alkaline conditions and cobalt at concentrations much higher than normally found in pulps *(22)*. At high pH (> 12) where PMS (SO_5^{2-}) is the predominant species, the decomposition rate decreases markedly and the sensitivity to copper disappears *(24)*.

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PMS Delignification Under Alkaline Conditions

The first evidence of the high reactivity of PMS with pulp under alkaline conditions is found in a paper showing that the rate of PMS reaction with lignin increases with pH *(25)*. This effect has also been observed for the epoxidation of olefins and attributed to the superior nucleophilicity of the dianion (SO_5^{-2}) *(26)*.

The reason this positive pH effect has not been shown before is probably related to the mode of addition of PMS on pulp. Since PMS stability is strongly affected by pH, preparing an alkaline PMS solution is not easily accomplished because it can decompose before it is used (the effective charge will therefore be much lower than expected). The present work is based on the development of a procedure to mix PMS, caustic and pulp together almost instantaneously which allows a reconsideration of the bleaching efficiency of PMS at high pH and the possibility of integrating such a treatment within an oxygen delignification process.

Experimental

Oxygen Delignification of Unbleached Pulp

The pulp sample (250g) was mixed at 10% consistency with $MgSO_4$ (0.5% on o.d. pulp) and NaOH (2.5% on o.d. pulp except when specified) in a Hobart mixer. The pulp was then rapidly heated in a microwave and put in a high shear mixer (Quantum, Mark IV). When the temperature reached 90°C, the reactor was pressurized to 690 kPa with oxygen. The pulp was mixed for 4 seconds at 2400 rpm, then mixed for 4 seconds every 30 seconds at 400 rpm for the specified reaction time. The pulp was either immediately treated or washed and stored in a cold room.

PMS Delignification of Unbleached Pulp

The source of PMS for the experiments was Oxone™ (Dupont) which is an acid salt mixture containing potassium peroxymonosulphate, and mono- and di-basic potassium sulphates. Because Oxone™ is strongly acidic in solution, NaOH had to be added to the pulp to neutralise the acidity of the PMS mixture and to give the appropriate alkaline pH after Oxone™ addition. The experiments on PMS delignification of unbleached pulp were done in plastic bags. Fifty grams of pulp was mixed at 10% consistency with a 6% caustic charge for 5 minutes and then left to sit for five minutes. At that time, 11.1 g of Oxone™ corresponding to 1% active oxygen (AO) on pulp was added (one active oxygen

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per HSO_5^{-1}). The suspension was hand-mixed for 10 minutes, and sporadically mixed thereafter for a total of 30 minutes. The pulp was then washed and the pH recorded. To compare the efficiency of *in-situ* DMD bleaching *(27)* and PMS bleaching, a control was treated with a mixture of PMS and acetone at the same AO charge by the method described previously *(25)*.

Oxygen and PMS in a Combined Stage

Whether the PMS addition was done at the beginning, during or at the end of the oxygen stage - (PxO), (OPxO), or (OPx) - the same addition procedure was followed. At the appropriate time, the Quantum mixer/reactor was opened and the caustic needed for pH adjustment was added to the pulp on one side of the reactor. The PMS charge was added on the other side as a powder and the reactor was resealed. The reactor was repressurized and heated if required. The pulp was subjected to 4 seconds of high-shear mixing and let to react.

Acid Wash (A) and Analytical Procedures

The acid stage was performed at 2% consistency for one hour at 50°C and followed by thorough washes with deionized water. The pH was adjusted to 1.5 using H_2SO_4. Kappa number, pulp viscosity and dry zero-span were measured according to PAPTAC Technical Standards G.18, G.24P, and D.27U respectively.

Results and Discussion

PMS Delignification Under Mild Alkaline Conditions

Our previous results *(25)* are summarized in Figure 1. The first two bars represent the delignification and viscosity of a black spruce kraft pulp which has been bleached using *in-situ* dimethyldioxirane (DMD) at pH 7.2. *In-situ* DMD bleaching means that acetone and PMS are added to the pulp buffered to pH 7.2 with sodium bicarbonate. DMD is generated during the stage. The three other sets of bars are from experiments where acetone has not been added (PMS bleaching) but the same active oxygen charge on pulp (1.5% A.O.) has been used. Figure 1 indicates that increasing the pH of PMS bleaching from 7.3 to 8.2 increased the delignification from 30% to 38% and the viscosity of the pulp by three units. These results led to the evaluation of using PMS to bleach pulp under alkaline conditions.

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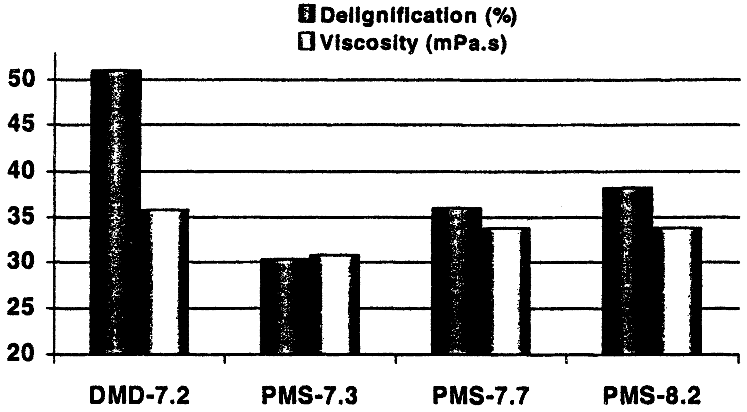

Figure 1. Delignification (%) and viscosity (mPa.s) obtained after bleaching of a softwood kraft pulp (kappa number, 28.9; viscosity, 38.5 mPa.s) using either in-situ DMD at pH 7.2 or peroxymonosulphate at pH 7.3, 7.7, or 8.2.

Effect of pH

A black spruce unbleached kraft pulp (kappa number, 27.6; viscosity, 39.6 mPa.s) was treated with PMS (1% A.O.) at room temperature. The pH of the pulp slurry was adjusted using different combinations of sodium bicarbonate, sodium carbonate and sodium hydroxide. In one case, acetone was added to generate DMD. Several observations can be made from the results presented in Table II:

- There is a significant increase of delignification when the pH is increased from 8.4 to 11 and delignification levels-off at pHs higher than 11.
- PMS bleaching at pH around 11 provides better delignification and similar viscosity than that obtained with a PMS/acetone mixture which generates DMD at pH 8.
- There is no significant viscosity loss during PMS bleaching up to a pH of 11. Higher pHs cause extensive viscosity loss. A selectivity decrease has also been reported for hydrogen peroxide bleaching at very high alkalinity *(28)* where pH is above the pKa of 11.6. Alternatively, the rapid mixing of a high charge of a strong acid (Oxone^{TM}) and a base (NaOH) with pulp may also have contributed to cellulose hydrolysis.

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Table II. Effect of pH on PMS delignification of a softwood kraft pulp.

pH	*Kappa number*	*Delignification (%)*	*Viscosity (mPa.s)*
Pulp	27.6	-	39.6
8.0*	18.9	31.5	33.7
8.4	21.3	22.8	33.9
9.6	19.1	30.8	33.3
11.0	16.8	39.1	31.0
12.4	16.0	42.0	21.0
12.7	17.3	37.3	20.1

*In that case, acetone was added to generate DMD.

From these results, it appears that efficient PMS delignification can be obtained using alkaline conditions similar to those encountered in an oxygen stage. Consequently, we looked at the possibility of combining oxygen delignification and PMS bleaching in a single stage, the goal being to boost the delignification achieved in an oxygen stage without requiring capital expenditure on a washer or a tower. The experiments were done in a high-shear mixer and we first assessed whether the mechanical treatment caused damage to the fibres.

Effect of High-Shear Mixing on Oxygen Delignified Pulp Strength

A softwood kraft pulp was exposed to different amounts of mixing at 90°C for 60 minutes with and without the oxygen delignification chemicals added. As described in Table III, the pulp was mixed with high shear (1200 rpm) for 4 seconds and then mixed at 400 rpm for 4 seconds every 30 seconds for the entire reaction time. At the end of the experiment, the pulp was washed and kappa number, viscosity, and zero-span tensile strength were determined.

A decrease of about 10% in the zero-span measurement was observed after the pulp was mixed with high shear whether the chemicals were added or not. It has been shown by Bennington and Seth (*29*) that pulp beating during high-shear mixing causes this effect.

Table III. Properties of a softwood kraft pulp mechanically treated in the absence or presence of oxygen delignification chemicals

	Kappa Number	*Viscosity (mPa.s)*	*Zero-span (km)*
Initial Pulp	24.7	31.3	16.8
No Chemicals	23.5	31.2	14.6
O_2 Delignification Chemicals	11.8	21.0	14.5

Table III shows that viscosity remained unaffected by mixing in the absence of chemicals while oxygen delignification decreased the pulp viscosity significantly without affecting the zero-span tensile strength. These results are in agreement with those of Bennington and Seth *(29)* who showed that there is no synergistic effect between mechanical and chemical treatment.

Different Modes of PMS Addition: (PxO); (OPxO); (OPx)

Three modes of PMS addition (1%AO change) were evaluated in the combined O_2/PMS stage where no washing was used between steps: These sequences are represented in Figure 2.

(PxO)	PMS was added at the beginning of the oxygen delignification stage. Total reaction time was 55 minutes but the reactor was opened after 25 minutes for sampling. After sampling, the reactor was repressurized and the pulp mixed with high shear again for 4 seconds.
(OPxO)	PMS was added to the oxygen stage after 25 minutes. This mode simulates a two-stage oxygen delignification system where PMS would be added just before the MC mixer of the second stage.
(OPx)	PMS was added at the end of the oxygen delignification stage. This mode simulates the process where PMS would be added in the blow line of the oxygen stage and allowed to react without further heating and repressurisation. The reaction was completed within 5 minutes.

In all cases, the pulp is treated for a total time of 55 minutes and submitted to two high-shear mechanical treatments. Only the points of addition of the chemicals (PMS, NaOH, and O_2) are different as indicated in Figure 2. The results are presented in Table IV where data from an oxygen delignification

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stage (O) and a PMS stage without oxygen (Px) have been included. Also included is an example where the pulp has been acid washed to remove transition metals prior to the stage: A(OPxO). Oxygen or PMS alone provided about 50% delignification with similar viscosity losses. Combining both chemicals in a single stage increased the delignification as compared to the oxygen stage alone. However, the results vary according to the mode of PMS addition selected. Adding PMS in front of the O stage (PxO) increased the delignification only from 49% to 63%. It seems that the oxidation of the lignin by PMS renders the residual lignin less susceptible to the slower attack by oxygen.

Adding PMS in between (OPxO) or at the end of the stage (OPx) was more efficient and provided in both cases 73% delignification. However, in the case of (OPxO), the cellulose was degraded and the pulp viscosity was low (13.3 mPa.s). Using an acid stage before (OPxO) increased the viscosity by reducing the transition metal ion content of the pulp but decreased the degree of delignification.

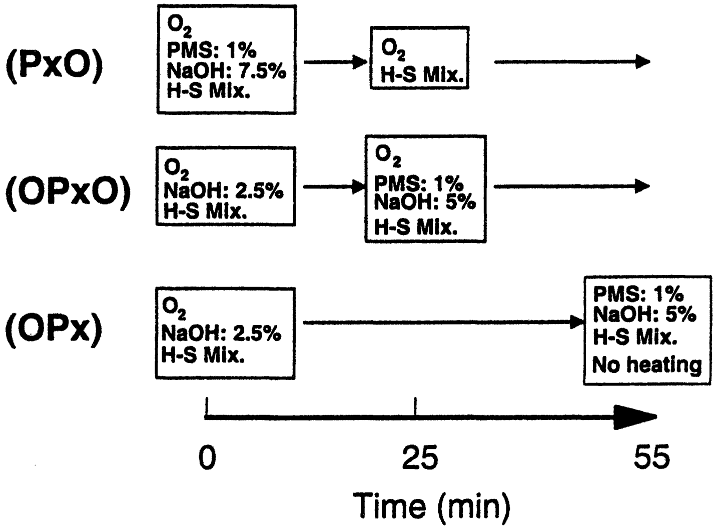

Figure 2. Sequence of chemical addition and high-shear mixing (H-S Mix) for the three modes of PMS addition in an oxygen stage.

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Table IV. Properties of a softwood kraft pulp treated in combined O_2 / PMS stages.

Sequence	*Kappa number*	*Delignification (%)*	*Viscosity (mPa.s)*	*Final pH*
Initial Pulp	24.7	-	31.3	-
O	12.7	48.6	20.8	12.2
Px	12.3	50.2	16.3	11.7
(PxO)	9.0	63.6	14.6	10.6
(OPxO)	6.6	73.3	13.3	9.8
A(OPxO)	7.5	69.6	15.2	9.3
(OPx)	6.6	73.3	17.3	10.0

NOTE: All the experimental conditions were identical except the addition point for PMS.

Lower delignification appears to be more likely the result of a decrease in the pH of the process than an effect of metal removal. In the case of (OPx), the high delignification level was obtained without inducing much carbohydrate damage. This mode gave a higher viscosity than with PMS alone (Px) probably because the pH of the PMS was lowered in the (OPx) stage. Based on these results, the (OPx) combined stage appears to be the most efficient and selective process and future effort will concentrate on this mode of addition.

Effect of pH on (OPx) Treatment of a Softwood Kraft Pulp

Data from Table 2 show that the optimal pH for a PMS delignification stage is around 11. It was necessary to confirm that this remained true in an (OPx) combined stage. An unbleached softwood kraft pulp (kappa number, 28.2; viscosity, 41.8 mPa.s) was oxygen delignified (2.5% NaOH, 0.5% $MgSO_4$) in the Quantum mixer for 60 minutes at 90°C. PMS (1% A.O.) was added after oxygen delignification as described previously and the NaOH charge was varied to give different pHs. The results are presented in Table V and in Figure 3.

As shown in the first row of Table V, the intermediate results at the end of the O-stages were very reproducible over 7 runs. The following rows indicate results for the combined (OPx) stages. The highest selectivity in the (OPx) process was obtained at pH 10.1; the delignification of this sample was almost 70%. The optimum pH for PMS reaction in the (OPx) stage appears to be a little bit lower that for a simple PMS stage. Figure 3 shows that there was a large increase in delignification when the pH was increased from 8.3 to 10.1 which corresponds to the change from HSO_5^{-1} to SO_5^{-2} (pKa 9.4). These results confirm

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the greater reactivity of the dianion toward lignin. This behaviour parallels results obtained with hydrogen peroxide which is also more reactive in the anionic form. Higher pHs do not provide any benefit in terms of delignification and damage the carbohydrates.

Table V. Effect of the pH of the PMS treatment on the overall efficiency of the (OPx) stage for a softwood kraft pulp.

Stage	*Final pH*	*Kappa Number*	*Viscosity (mPa.s)*	*Selectivity (V/K)*
O_2	12.1 ± 0.2	15.2 ± 0.3	22.6 ± 0.7	-
(OPx)	2.1	13.6	15.0	1.10
	3.5	11.9	16.9	1.42
	8.3	11.0	18.9	1.72
	10.1	8.6	16.0	1.86
	11.9	8.2	13.0	1.58
	12.5	8.2	11.5	1.40
	12.9	7.9	10.3	1.30

NOTE: Selectivity is expressed as the ratio of viscosity over kappa number (V/K).

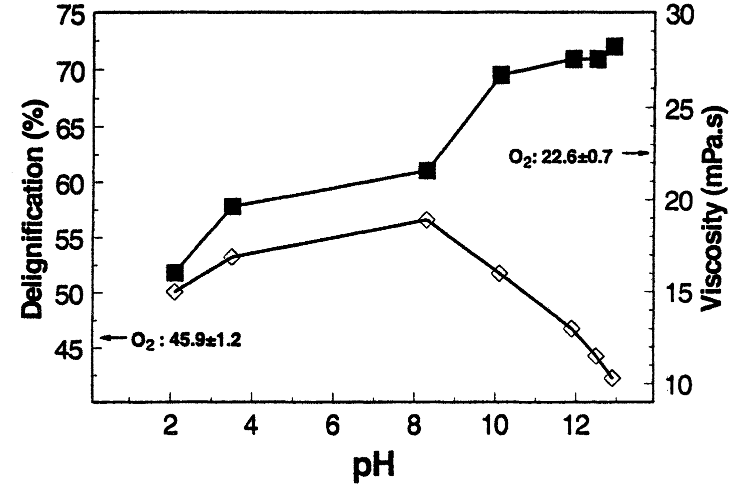

Figure 3. Delignification (■) and viscosity (◇) as a function of the final pH of the OPx stage. Intermediate delignification and viscosity after oxygen are indication (by arrows) on both axes

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We made a comparison between the efficiency of the (OPx) process and of an (OP) process where hydrogen peroxide is

used instead of PMS under the same conditions. An unbleached black spruce kraft pulp (kappa number, 28.2; viscosity, 41.8 mPa.s) was oxygen delignified (2.5% NaOH, 95°C, 55 min) and a 1% active oxygen charge of either PMS or H_2O_2 was added to the pulp following the procedure described previously. Pulp samples were taken after 30 and 90 minutes of the PMS or the H_2O_2 treatment. The results are shown in Table VI.

Table VI. Comparison of (OPx) and (OP) (1% A.O. charge) delignification of a softwood kraft pulp.

	O_2	*Px*		*O_2*	*P*	
Time (min)		*30*	*90*		*30*	*90*
Kappa Number	18.4	11.0	10.8	18.1	16.8	16.5
Delignification (%)	35	61	62	36	40	41
Viscosity (mPa.s)	28.2	20.6	20.1	27.6	26.7	26.4
Peroxide Residual		tr.	tr.		tr.	tr.
pH	11.6	9.7	9.8	11.8	11.5	11.6
Temperature (°C)	90	66	48	90	64	47

The (OPx) process increased the delignification from 35 to 62% while hydrogen peroxide had very little effect on kappa number. It appears that the PMS reaction with pulp is complete after 30 minutes. Despite little delignification, hydrogen peroxide was consumed rapidly. Viscosity was maintained which suggests that peroxide was not lost by decomposition into harmful radicals. Rather than decomposition, it is more likely that the H_2O_2 is consumed by organic material in the filtrate. PMS and hydrogen peroxide residual concentrations were negligible after 30 minutes.

Potential and Prerequisite for Industrial Application

Adding large quantities of Oxone™ (PMS) and caustic in the O stage is not economic and such a process has little chance of being applied industrially. However, research conducted at Paprican *(24,30)* has shown that it is possible to produce PMS in satisfactory concentration by catalytic oxidation of sodium sulphite with molecular oxygen under alkaline conditions. Practical yields appear to be possible.

Using such a generator will minimize the cost of PMS and will allow the injection of an alkaline PMS stream directly into the blow-line of an oxygen reactor. The effect of large amount of sodium sulphate in the oxygen effluent is also presently been examined and will be the basis of a future report.

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Conclusions

- With the appropriate mode of addition and mixing, peroxymonosulphate (PMS) can be used to selectively delignify softwood kraft pulp under alkaline conditions. PMS is at its most reactive under such conditions.
- PMS delignification can be combined with oxygen delignification in a single stage without any washing between steps.
- The most selective process (OPx) was obtained when PMS was added at the end of the O stage. This process would be achieved industrially by adding PMS to the blow-line.
- Delignification higher than 70% has been obtained with softwood kraft pulp in a combined (OPx) stage with a residence time of 55 minutes.

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Chapter 9

Bleaching of High-Yield Lignocellulosic Pulps with Peroxygen Reagents

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A novel bleaching method for mechanical pulps has been developed that uses a conventional bleaching chemical, hydrogen peroxide, in combination with peracetic acid (Pa), peroxymonosulfate (Ps), or dimethyldioxirane (DMD). There are two main benefits from using Pa, Ps and DMD as an alternative to hydrogen peroxide: enhanced bleaching efficiency and reduced pulp yield loss. In bleaching wood pulps, the yield improvement is the main benefit. For wheat straw mechanical pulp, the novel bleaching method results in both an increased brightness and higher pulp yields.

The objective in the bleaching of mechanical pulps is to selectively remove color-contributing components (chromophores) while simultaneously preserving pulp yield. In current practice, this involves mainly the use of hydrogen peroxide especially when high brightness is targeted. The hydrogen peroxide bleaching of mechanical pulps is usually performed in alkaline media that promote the dissociation of hydrogen peroxide to perhydroxyl ion (HOO^-), generally accepted as being the principal active species in hydrogen peroxide bleaching systems. This anion is a strong nucleophile that, during bleaching, preferentially attacks conjugated carbonyl groups and olefinic double

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

bonds such as those found in cinnamaldehyde and quinonoid structures, but not electron-rich aromatic rings (*1*). Consequently, chromophores are removed while the extent to which the rest of the lignin macromolecule is attacked is limited (*2*). Likewise, the degradation of carbohydrates is essentially a minor reaction when the decomposition of hydrogen peroxide is efficiently controlled (*1*). Therefore, bleaching occurs without a significant impact on pulp yield.

Nevertheless, mechanical pulps cannot be bleached at an acceptable cost to the same brightness as bleached chemical pulps (above 90 ISO). In bleaching mechanical pulps, with increasing hydrogen peroxide dosage brightness response is rapid, then slows down, and finally reaches a plateau from which further increasing hydrogen peroxide charge only slightly increases the brightness (*3*). In general, high hydrogen peroxide charge coupled with high alkalinity is essential to achieve high pulp brightness, and the major part of the added bleaching chemical is consumed in attaining the last few points of brightness gained. On the other hand, bleaching reactions involving lignin modification and/or solubilization are inevitably accompanied by removal of carbohydrates, thereby resulting in pulp yield loss.

Although the cause of the brightness ceiling in the hydrogen peroxide bleaching of mechanical pulps is not established, it is presumed that certain chromophores present in pulps are resistant to the perhydroxyl ion. The nature of these chromophores varies from one lignocellulosic species to another. In fact, pulps from different fiber sources respond differently to hydrogen peroxide bleaching. Generally, the softwood pulps are bleached by hydrogen peroxide to a brightness of up to 80 ISO and the hardwood pulps to a brightness of up to 85 ISO. However, when equivalent amounts of bleaching chemicals are applied the brightness of wheat straw pulp is far lower than the above-mentioned levels (*4*). This indicates that wheat straw contains greater quantities of peroxide-resistant chromophores. To overcome the limitation of hydrogen peroxide bleaching, it could be beneficial to use other bleaching chemicals that could help reach a higher brightness. An example of the combination of two oxidants is that pressurizing mechanical pulp with oxygen during alkaline peroxide bleaching increases its brightness by 1–2 points (*5*). This additional brightness gain could be due to the elimination of stilbenes by oxygen (*6*).

The present investigation aims at evaluating peracids as alternatives to hydrogen peroxide in the mechanical pulp bleaching. Peracids may be regarded as activated derivatives of hydrogen peroxide. The active oxygen of hydrogen peroxide can be transformed to an active peroxy acid form by direct oxidation of an acid, such as acetic acid or sulfuric acid, with hydrogen peroxide, to form peracetic acid or peroxymonosulfuric acid (also known as Caro's acid). The higher oxidizing power of these peroxy acids allows them to react with lignin to a greater extent or to be used in bleaching reactions under more moderate conditions as compared to hydrogen peroxide (*7*). While being versatile and acting as

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both electrophilic and nucleophilic agents in bleaching reactions, depending upon the reaction media chosen, peracids have mainly been studied as delignifying and bleaching reagents in the chlorine-free bleaching of chemical pulps (*8*). However, the mechanical pulp bleaching involves mainly nucleophilic reactions and lignin-retaining bleaching (*1*). The use of peracetic acid in mechanical pulp bleaching has been a research subject, but has not received much attention. In 1965, peracetic acid was evaluated as a bleaching agent for mechanical pulps at neutral pH (*9*). Ultraviolet and infrared spectral studies of the bleached pulps have shown that bleaching results primarily from modification of some lignin side chain groups (α,β-unsaturated aldehydes, conjugated double bonds and α-carbonyl groups), similar to the reactions that occur in hydrogen peroxide bleaching (*10*). Recently, Li *et al* showed that adding peracetic acid to either the first or second stage in a two-stage peroxide bleaching sequence enhanced the brightness response of a spruce groundwood pulp (*11*). On the other hand, no attention has been paid to the use of peroxymonsulfuric acid on mechanical pulps, probably due to its strong oxidation potential that does not fit into the traditional bleaching concept for mechanical pulps. Moreover, dimethyldioxirane (DMD), which can be generated from the reaction of peroxymonosulfate with acetone, is a strong electrophile (*12*) and is a very effective bleaching agent for chemical pulps (*13*, *14*).

The objective of the present work was to evaluate the effectiveness of peracetic acid, peroxymonosulfuric acid, and DMD as novel bleaching agents in bleaching wood and wheat straw mechanical pulps. The effects of bleaching parameters, including pH, dosage, temperature and time, on the brightness response were evaluated. The sequential use of the new bleaching chemicals with hydrogen peroxide was also investigated.

Materials and Methods

Aspen CTMP of 54.4 ISO was supplied by a local pulp mill. Spruce TMP of 54.5 ISO was produced at our pilot plant. Three wheat straw mechanical pulps were used in this work: pulp A of 40.9 ISO (refined following 2 % H_2O_2 alkaline peroxide impregnation), pulp B of 52.3 ISO (refined following 4 % H_2O_2 alkaline peroxide impregnation), and pulp C of 35.9 ISO (RMP).

Prior to bleaching, the pulps were diluted and disintegrated with mechanical stirring. A solution of N-(2-hydroxyethyl)ethylenediaminetriacetic acid (HEDTA) at a charge of 0.5 % on o.d. pulp was then added to the pulp suspensions. The chelation was performed at about pH 5, 60 °C and 2 % pulp consistency for 30 min with stirring. The pulps were then filtered in a Buchner funnel and washed thoroughly with water.

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All bleaching trials were performed in polyethylene bags. The bleaching chemicals were commercial products and the charges given on the basis of o.d. pulp. Hydrogen peroxide trials using varying amounts of H_2O_2 and NaOH were carried out at 15 % pulp consistency, 60 °C for 3 hours. The other chemical charges were: 3 % Na_2SiO_3, 0.05 % $MgSO_4$, 0.2 % DTPA. Peracetic acid (Pa) bleaching trials employed an equilibrium peracetic acid mixture consisting of about 32 % peracetic acid and less than 6 % hydrogen peroxide. The Pa bleaching consistency, temperature and time were the same as in the hydrogen peroxide bleaching. The source of peroxymonosulfate (Ps) was the triplet salt, $2KHSO_5$-$KHSO_4$-K_2SO_4 (OXONE, DuPont). In all the Ps bleaching trials except for the pH effect experiment, pH was buffered in the 7.0 – 7.8 range with $NaHCO_3$ (10 – 15 % on pulp depending on Ps charge). Washing was applied between stages.

The concentration of hydrogen peroxide, peracetic acid, and peroxymonosulfate was determined iodometrically. Bleached pulps were diluted with water and acidified to about pH 5 by sodium metabisulfite prior to preparation of brightness pads. Brightness values were recorded in a Technibrite Micro TB-1C instrument. Pulp yields were calculated from gravimetric measurements using pulp consistency and total weight before and after bleaching. The standard deviation was ±0.5 %.

Ferulic acid, ethyl ferulate and 3, 4-dimethoxycinnamic acid were commercial products and employed as received. 5, 5'-diferulic acid was synthesized according to Drumond *et al* (*15*) and Ralph *et al* (*16*) with modifications. The experimental procedures in oxidation and sample preparation were similar to those reported previously (*17*). The reactions were carried out using a 4 mole ratio of active oxygen to substrate at ambient temperature (22 °C) for 3 h. The reaction pH was kept in the range of 7.0 – 7.8 by adding sufficient amount of $NaHCO_3$. The Ps and DMD reactions used, respectively, 50 % ethanol and acetone aqueous solution as solvents. The amount of remaining substrates was determined by HPLC at 280 nm according to Pan *et al* (*18*).

Results and Discussion

Pulp Bleaching Performance

pH Effect on Brightness Response

As mentioned earlier, the mechanism of mechanical pulp bleaching involves mainly nucleophilic reactions with lignin. The formation of an adequate concentration of nucleophiles in bleaching systems, such as perhydroxyl (HOO^-),

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peracetate ($CH_3CO_3^-$) or peroxymonosulfate (SO_5^-) ions, is essential to the bleaching action of hydrogen peroxide, peracetic acid and peroxymonosulfuric acid, respectively. The dissociation of these three bleaching chemicals into their corresponding anions increases as the pH approaches the pKa in the direction from low to high. Accordingly, their bleaching activity is highly pH-dependent. The pKa at 25 °C is 11.6, 8.2 and 9.3 (*19*, *20*), respectively, for hydrogen peroxide, peracetic acid and peroxymonosulfuric acid. Therefore, in practice hydrogen peroxide bleaching requires a strong alkaline medium, and peracetic acid or peroxymonosulfuric acid bleaching is preferably performed under neutral to moderately alkaline conditions.

Figure 1 shows the effect of pH on the brightness of aspen and wheat straw pulps bleached with peracetic acid or peroxymonosulfate. In agreement with theory, the best brightness development for both peracetic acid and peroxymonosulfate is obtained at a pH range of neutral to moderately alkaline. On the other hand, the consumption of the bleaching reagents increases with the pH. This is not surprising because the decomposition of the peroxy acids increases with pH up to a maximum rate when pH is equal to their pKa (*20*).

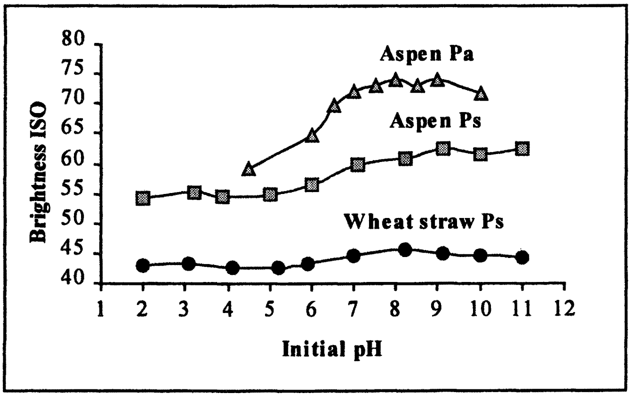

Figure 1. Brightness response of aspen CTMP and wheat straw pulp A treated with peracetic acid (Pa) and peroxymonosulfate (Ps) at varying pH; Pa: 2 %; Ps: 1 % as active oxygen, 5 % pulp consistency, 22 °C for 3 h.

In this work, only the neutral experiment (at about pH 7) employed a buffered pH. In the rest of the experiments, the pH dropped as the bleaching reactions proceeded. Comparatively, the pH drop was greater in the peroxymonosulfate bleaching than in the peracetic acid bleaching since in the former case a strong acid, sulfuric acid, is generated. As shown in Figure 1, when the initial pH was above 9 the brightness response declined in the peracetic acid bleaching. This can be attributed to accelerated decomposition of peracetic acid leading to a substantial waste of the bleaching agent. In contrast, the peroxymonosulfate bleaching provided a maximum brightness increase at a broader initial pH range from neutral to alkaline. This observation can be explained by the higher pKa of peroxymonosulfuric acid and the more rapid pH decrease of the bleaching solution.

The alkali source can be sodium hydroxide, sodium carbonate, or sodium bicarbonate. Sodium hydroxide provides a high initial pH, but the two other alkalis provide more buffering. Our experiments showed that the best bleaching efficiency was achieved for peracetic acid and peroxymonosulfate when the bleaching reactions started at a pH close to their pKas (8.2 and 9.3). There was little difference in brightness response between the bleaching experiments where the pH was held constant and those in which the pH was allowed to decrease during bleaching. Nevertheless, for the sake of comparison the following bleaching experiments of peracids were performed at a pH range of 7.0 – 7.8 buffered by $NaHCO_3$.

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Effect of Peroxymonosulfate Charge and Acetone Addition

Table I shows the brightness development of aspen and wheat straw pulps in peroxymonosulfate pre-bleaching (Ps) and subsequent hydrogen peroxide bleaching (P). It can be seen that the two pulps respond differently to this bleaching sequence, possibly due to the difference in the nature of chromophore species present in the pulps. In the case of aspen pulp, as the dosage of peroxymonosulfate increases the brightness increases steadily and the yellowness (b*) decreases in the Ps stage. However, the brightness gain and yellowness reduction achieved in the Ps stage were not retained in the subsequent P stage. As a result, there was little difference in brightness and yellowness when the pulps were fully bleached with the Ps-P sequence.

A similar trend of the change of brightness response in the Ps stage was observed with wheat straw pulp, but the yellowness change in the Ps stage was only marginal. In contrast to the case of aspen pulp, the brightness gain by the Ps bleaching was carried over to the P stage. It was clearly demonstrated that increasing the Ps charge significantly raises the brightness and lowers the yellowness for the fully bleached pulp. This observation would suggest that the chromophore structures in aspen and wheat straw have different reactivities toward the bleaching agents. It seems that wheat straw contains considerable amounts of chromophores resistant to hydrogen peroxide so that a synergy can be obtained from combining the two oxidizing reagents. On the other hand, no

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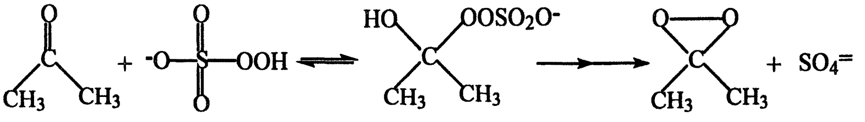
synergistic effect was seen when aspen pulp was bleached with peroxymonosulfate and hydrogen peroxide in sequence. It is likely that the two bleaching chemicals react with the pulp in a similar manner.

Table I. Effect of Peroxymonosulfate Charge and Acetone Addition on the Bleaching Performance of a Ps-P Sequence; Ps: 5 % pulp consistency, room temperature (22 °C) for 3 h.; P: 4 % H_2O_2 and 4 % NaOH

Ps % as active oxygen	*Aspen CTMP*				*Wheat Straw Pulp A*			
	Brightness		b*		Brightness		b*	
	Ps	Ps-P	Ps	Ps-P	Ps	Ps-P	Ps	Ps-P
0		80.9		8.26		50.1		23.4
No acetone added								
0.2	57.4	81.7	15.0	7.91	43.4	53.0	23.9	22.2
0.5	59.7	82.1	14.5	7.76	44.6	54.9	23.8	21.0
1.0	61.8	81.6	14.2	7.89	46.2	56.5	23.9	20.6
2.0	64.5	81.7	13.6	7.82	48.2	57.0	23.2	20.3
5 mole ratio of acetone to active oxygen								
0.2	58.8	82.0	14.6	7.93	43.2	53.9	24.5	21.8
0.5	60.7	81.7	14.7	8.13	44.6	56.7	24.7	20.3
1.0	61.3	81.1	15.7	8.93	46.5	58.3	24.6	18.9
2.0	59.5	78.8	18.2	10.5	49.2	60.6	24.0	17.5
10 mole ratio of acetone to active oxygen								
0.2	58.6	82.3	14.6	7.76	43.4	55.5	24.4	21.0
0.5	60.4	82.2	14.9	8.10	44.8	57.3	24.6	19.9
1.0	60.7	81.1	16.4	8.98	46.6	59.5	24.6	18.6
2.0	58.7	79.2	18.9	10.5	48.8	62.7	24.3	16.8

Recently, dimethyldioxirane (DMD) has been reported to be a highly selective delignification agent (*13*, *14*). Dioxiranes are a powerful class of electrophilic oxidants that can react with electron rich carbon-carbon double bonds in aliphatic side chain to form epoxides or in aromatic rings to form arene oxides (*12*, *21*). These epoxides are readily hydrolyzed to diols in aqueous solution. The most common current method of preparing dioxiranes involves the oxidation of ketones by peracids, particularly peroxymonosulfuric acid (*12*). So, DMD is a product of the reaction between peroxymonosulfate and acetone, as described in the equation shown below. It is important to maintain the pH at 7.0 – 7.5 by means of bicarbonate or phosphate buffer for the generation of DMD (*12*, *21*). In pulp bleaching, DMD can be added as an isolated form by reacting peroxymonosulfate with acetone in a buffer and then distilling off the DMD as an acetone solution or generated *in situ* by mixing acetone and buffer with pulp then adding the required amount of peroxymonosulfate (*13*, *22*, *23*). The use of *in situ* generated DMD in pulp bleaching is probably more efficient and practical due to the low yield and instability of isolated DMD (*13*, *22*).



In the present work, the bleaching activity of *in situ* generated DMD on mechanical pulps was investigated. To do this, different amounts of acetone were added to the Ps stage at buffered pH. Given a charge of active oxygen, the amount of added acetone may be an important factor influencing the bleaching efficiency of *in situ* DMD. In the previous reports (*13*, *22*, *23*, *24*), a broad range of acetone-to-active oxygen ratio was used in chemical pulp bleaching. A minimum amount of acetone must be applied to allow the formation of an adequate concentration of DMD. Similarly, efficient mixing of pulp with acetone and buffer is essential to optimizing the *in situ* DMD bleaching (*13*). Lee *et al* (*22*) chose a 1.5 mole ratio of acetone-to-active oxygen, but Ragauskas used a much greater ratio (*13*).

In our preliminary study, acetone was added to the Ps stage at an acetone-to-active oxygen mole ratio of 1.5 and the acetone addition had little effect on the brightness. It was further observed that the bleaching activity increased with increasing the ratio of acetone-to-active oxygen. To obtain a significant difference for comparison, relatively greater ratios are needed to generate sufficient amounts of DMD. Table I gives the results obtained from the bleaching trials using 5 and 10 mole ratios of acetone-to-active oxygen. To address possible effects of increased acetone addition on bleaching efficiency, control runs were conducted without peroxymonosulfate and little effect on the brightness of Ps and Ps-P was observed.

As shown in Table I, aspen and wheat straw pulps behave differently toward the DMD bleaching. In the case of aspen pulp, small amounts of DMD slightly increase the Ps brightness, but do not have impact on the Ps-P brightness. As the charge of DMD was increased by applying more peroxymonosulfate and more acetone, the pulp yellowed as revealed by lower brightness and higher b*. This adverse effect of DMD can be more clearly seen on the Ps-P bleached pulp in which the final brightness was pronouncedly decreased and the yellowness raised. By contrast, the DMD bleaching had a positive effect on wheat straw pulp. The effectiveness was more evident for the Ps-P bleached pulp. The results obtained here again suggest that the nature of chromophore species is quite different between aspen and

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wheat straw. As a strong electrophilic agent, DMD may attack aromatic structures in aspen lignin and create new chromophores, thereby yellowing the pulp. On the other hand, DMD's powerful oxidizing activity helps destroy peroxide-resistant chromophores present in wheat straw and results in substantial brightness increase.

Reactivity of Oxidants

Figures 2 gives a comparison of the reactivity of peroxymonosulfate and DMD with wheat straw pulp. A similar trend was observed on aspen pulp. In general, DMD was more reactive than peroxymonosulfate, as demonstrated by more rapid and greater consumption. Similarly, in the DMD bleaching the brightness increased and reached a plateau in a short period of reaction time. When the pulp was bleached with peroxymonosulfate, the brightness increased more gradually and the bleaching action continued for a longer time. This reactivity difference allows the two bleaching reagents to perform bleaching in different manners. Generally, the DMD bleaching requires shorter retention time or lower temperature, whereas the peroxymonosulfate bleaching requires longer retention or higher temperature.

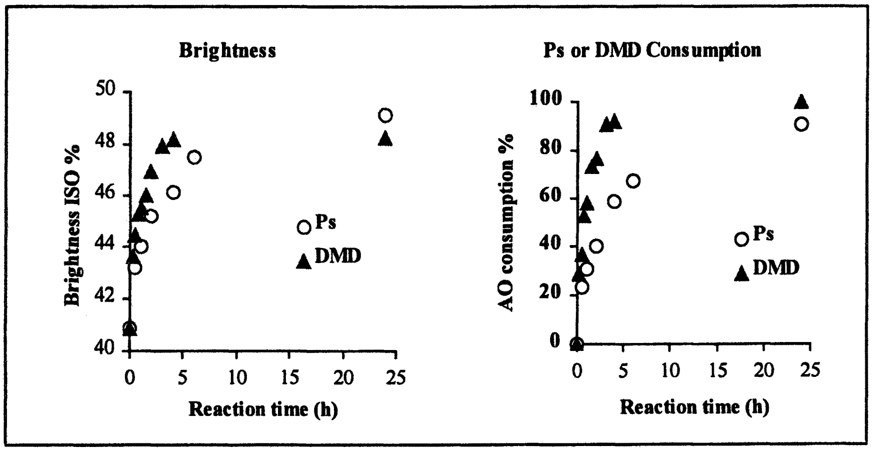

Figure 2. Difference in bleaching reactivity of peroxymonosulfate and in situ generated DMD with wheat straw pulp A; 1 % Ps (as active oxygen), 5 % pulp consistency, room temperature (22 °C); 5 mole ratio of acetone for DMD

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Bleaching Parameters

In this section, several key process parameters are assessed in order to find out optimum treatment conditions of peroxymonosulfate and DMD bleaching. For a given charge of bleaching chemicals, raising the pulp consistency increases the concentration of bleaching agents and thus results in greater brightness gain. This is clearly seen in Figure 3.

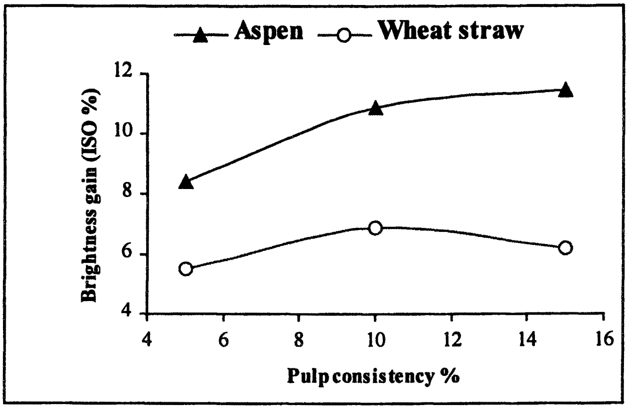

Figure 3. Effect of pulp consistency on brightness response in peroxymonosulfate treatment of aspen pulp and wheat straw pulp A; 1 % Ps (as active oxygen), room temperature (22 °C) for 4 h.

Figure 4 shows the changes in brightness and consumption of active oxygen when aspen pulp was bleached with peroxymonosulfate. It can be seen that the temperature had little effect on the brightness, but only determined the time to reach the maximum brightness. Similar results were observed on wheat straw pulp. The temperature and time are interchangeable, and an increase in bleaching temperature can compensate for a reduction in retention time and vice versa. This character gives peroxymonosulfate bleaching more flexibility to fit into current bleaching operations.

It appears that the DMD bleaching is quite sensitive to temperature and time. As shown in the preceding section, DMD is very reactive and consumed quickly. In Figure 5 we can see that at 60 °C the brightness of wheat straw reaches a maximum and the oxidant is all consumed in a short period of time. As the treatment continued from this point the brightness decreased remarkably. For the three temperatures studied here, the treatment at 40 °C for 30–60 min offered the best brightness

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response. This observation herein as well as that in the preceding section would suggest that DMD bleaching is preferably carried out at relatively low temperature.

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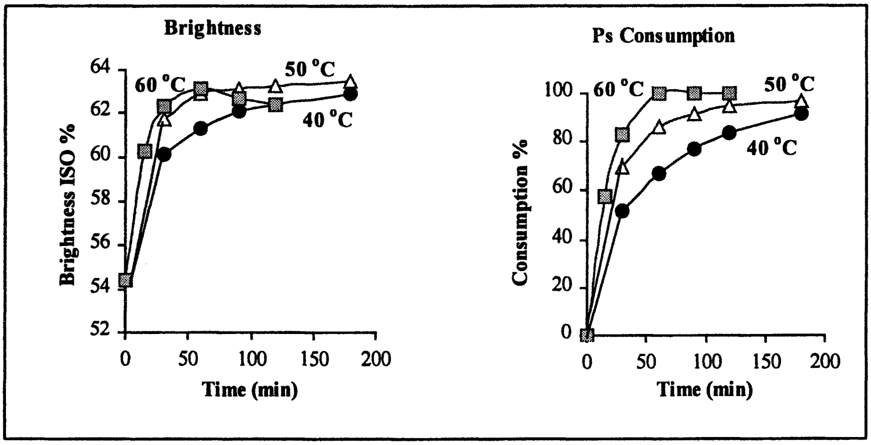

Figure 4. Effect of temperature and time on brightness response in the Ps treatment of aspen pulp; 0.5 % Ps (as active oxygen), 10 % pulp consistency

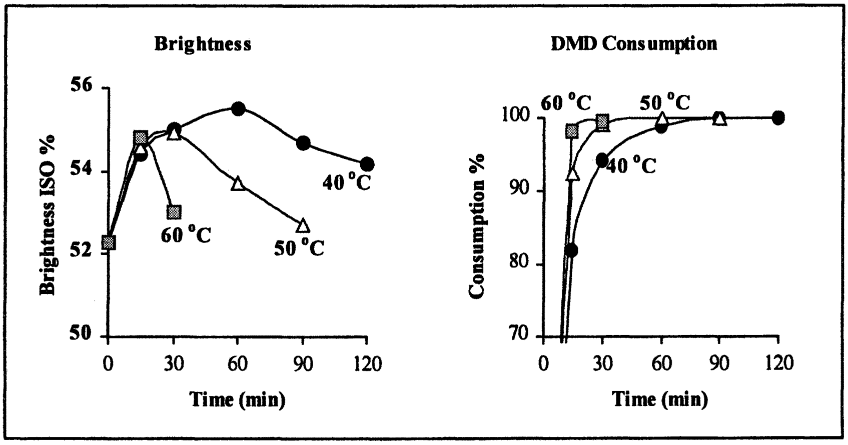

Figure 5. Effect of temperature and time on brightness response in the in situ generated DMD treatment of wheat straw pulp B; 0.5 % Ps (as active oxygen), 5 mole ratio of acetone, 10 % pulp consistency

Comparison of Various Bleaching Scenarios

Figure 6 shows a comparison of the brightness response of aspen pulp with the three bleaching chemicals. At equivalent amounts of oxidants, the peracetic acid bleaching provides a slightly greater brightness increase than does the hydrogen peroxide bleaching. This is in agreement with the earlier observation of Li *et al* on a spruce groundwood pulp (*11*). The peroxymonosulfate gives the smallest brightness increase. However, at equivalent charges, the bleaching efficiency of hydrogen peroxide is much higher than that of peracetic acid when one realizes that the molecular weight of peracetic acid (78) is more than double that of hydrogen peroxide (34). Considering the cost of the peracids, it would be more realistic to use peracetic acid or peroxymonosulfate in a small dosage in combination with hydrogen peroxide. The subject of this section is to investigate possible complementary or synergistic effect between hydrogen peroxide and peracetic acid or peroxymonosulfate or DMD when they are used sequentially. Table II summarizes the bleaching performance of a number of bleaching scenarios for aspen, spruce and wheat straw pulps.

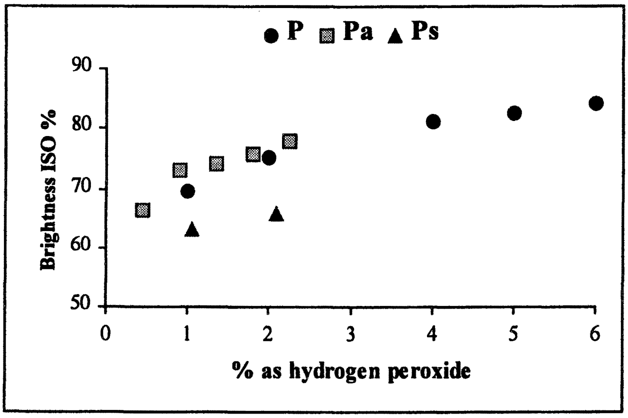

Figure 6. Comparison of brightness response in the aspen pulp bleaching with hydrogen peroxide (P), peracetic acid (Pa) and peroxymonosulfate (Ps); Ps bleaching: 15 % pulp consistency, room temperature (22 °C) for 4 h.

Overall, no synergy was observed between P and Pa or between P and Ps for the wood pulps. With the same total bleaching chemical charge, similar brightnesses were achieved when part of the peroxide is replaced by Pa or Ps.

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The addition order of the chemicals did not affect the final brightness. On the other hand, for wheat straw pulp the combination of Ps or DMD with P did show synergy and substantially improve the brightness increase. Consistent with the observations presented in the previous sections, the results indicate that the use of Ps or DMD in combination with P has a cooperative effect in bleaching wheat straw pulp. The results in Table II also show that it is more effective to place Ps or DMD prior to P stage and the reverse order causes a brightness decrease of 2 ISO units. This indicates that the Ps or DMD bleaching may create new chromophores that can be eliminated by subsequent hydrogen peroxide bleaching.

Table II. Comparison of Brightness Values of Aspen, Spruce and Wheat Straw Pulps Bleached by Various Bleaching Sequences; for aspen and spruce bleaching the Ps charges are given as active oxygen; for wheat straw bleaching the Ps or *in situ* DMD charges are given as H_2O_2

Aspen Pulp (55.5 ISO)	*Pre-bleaching*	$P_{1\%}P_{4\%}$ - 83.1 ISO $Pa_{1\%}P_{4\%}$ - 82.9 ISO $Ps_{1\%}P_{4\%}$ - 82.5 ISO
$P_{4\%}$ - 80.9 ISO $P_{5\%}$ - 82.6 ISO $P_{6\%}$ - 83.8 ISO	*Inter-stage bleaching*	$P_{2\%}P_{4\%}$ - 83.8 ISO $P_{1\%}Pa_{1\%}P_{4\%}$ - 83.5 ISO $P_{1\%}P_{4\%}$ - 83.5 ISO $P_{1\%}Ps_{0.2\%}P_{4\%}$ - 83.8 ISO $P_{1\%}Ps_{0.5\%}P_{4\%}$ - 84.2 ISO
Spruce Pulp (54.5 ISO)	$P_{4\%}$ - 75.2 ISO $Ps_{1\%}P_{4\%}$ - 76.9 ISO	
Wheat Straw Pulp C (37.7 ISO)	*Pre-bleaching*	$Ps_{2\%}P_{4\%}$ - 56.0 ISO $(DMD)_{2\%}P_{4\%}$ - 57.2 ISO
$P_{4\%}$ - 51.2 ISO	*Inter-stage bleaching*	$P_{2\%}(DMD)_{2\%}P_{2\%}$ - 58.4 ISO
$P_{6\%}$ - 54.2 ISO $P_{2\%}P_{4\%}$ - 54.7 ISO	*Post-bleaching*	$P_{4\%}Ps_{2\%}$ - 53.9 ISO $P_{4\%}(DMD)_{2\%}$ - 55.2 ISO

Pulp Yield Conservation

As a general rule, mechanical pulp bleaching involves lignin-retaining bleaching reactions and should not cause significant pulp yield loss. In an ideal scenario, bleaching agents would selectively remove chromophoric groups and not attack the lignin macromolecular structure. This may be the case for

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hydrosulfite bleaching that results in a quite limited brightness increase. In hydrogen peroxide bleaching, especially when high brightness is targeted, lignin dissolution inevitably occurs to a certain extent. It is well known that a small amount of an applied bleaching chemical gives an initial rapid brightness development and the final brightness gain of several points is attained at the expense of consuming the majority of the peroxide (*3*). It is presumed that delignification is quite likely involved in the removal of chromophores in the final phase of brightness development in hydrogen peroxide bleaching. We found that with increasing hydrogen peroxide and alkali charge the yield loss versus the brightness increase becomes more dramatic and hemicelluloses tend to dissolve to a greater extent than lignin (*unpublished results*). This is not surprising because hemicelluloses are an alkali-labile component.

Figure 7 shows the changes in brightness and yield as a function of NaOH charge when aspen pulp or wheat straw was bleached with hydrogen peroxide. It can be seen that the dissolution of pulp components accompanies the brightness development. It is also found that the yield loss is proportional to the increase in alkali charge irrespective of the hydrogen peroxide charge. In comparison, softwood, such as spruce, has a different profile from those in Figure 7. The yield loss is less pronounced probably because spruce contains less hemicelluloses. The brightness is more sensitive to the alkali charge; too high an alkalinity causes alkali-darkening reactions to offset brightness increase.

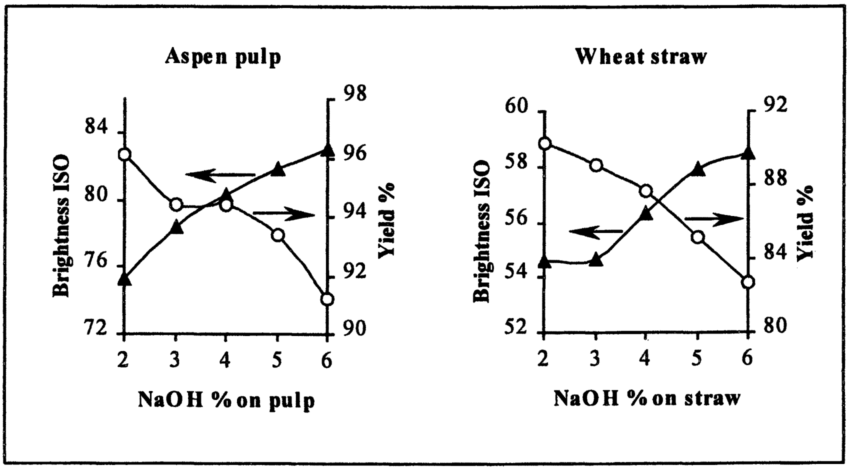

Figure 7. Changes in brightness and yield as a function of NaOH charge when aspen pulp and wheat straw are bleached with 4 % and 6 % H_2O_2, respectively

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In practice, applying a high peroxide charge with sufficient alkalinity is essential to attaining a high brightness. As discussed above, this common bleaching practice causes significant pulp yield loss. Obviously, hydrogen peroxide has limitations in bleaching mechanical pulps. On the other hand, this gives an opportunity for peracids to be an alternative to hydrogen peroxide in mechanical pulp bleaching. There are two scenarios in which peracids can help overcome the limitations of hydrogen peroxide. First, peracids could selectively attack peroxide-resistant chromophores. Secondly, as demonstrated in the preceding sections, bleaching with the peracids is carried out under neutral or mildly alkaline conditions such that the dissolution of lignin and hemicelluloses in bleaching can be minimized. Tables III and IV give examples of yield improvement that could justify a partial peracid substitution for hydrogen peroxide in the mechanical pulp bleaching. Overall, the pulp yield loss resulting from treatment with peracetic acid, peroxymonosulfate or DMD is much smaller than the loss resulting from hydrogen peroxide treatment. Partially replacing hydrogen peroxide by equivalent amount of peracetic acid or peroxymonosulfate in a bleaching sequence increases or maintains brightness while noticeably improving the pulp yield. The yield conservation can be more clearly seen for wheat straw in Table IV in which the two- or three-stage bleaching sequences were done with the same equivalent of bleaching chemicals. It is again shown that in bleaching wheat straw pulp the use of peroxymonosulfate or DMD in combination with hydrogen peroxide increases both the brightness and the yield.

Table III. Comparison of Yield of Aspen Pulp after Different Bleaching Treatments; initial pulp brightness of 55.1 ISO; Ps dosage as active oxygen; in P stage H_2O_2/NaOH of 1

Bleaching method	*Pa vs. P*		*Ps vs. P*			
	$P_{4\%}$	$Pa_{4\%}$	$P_{6\%}$	$Ps_{0.5\%}$	$Ps_{0.5\%}$ $P_{5\%}$	$Ps_{1\%}$ $P_{5\%}$
Brightness	80.3	77.7	84.4	63.2	84.2	84.3
Yield (%)	93.9	97.6	89.5	99.0	92.0	91.0

Table IV. Comparison of Yield of Wheat Straw Pulp C after Different Bleaching Treatments; initial pulp brightness of 37.7 ISO; Ps or *in situ* DMD dosage as H_2O_2; in P stage H_2O_2/NaOH of 1.3

	$P_{6\%}$	$Ps_{2\%}$	$DMD_{2\%}$	$P_{2\%}P_{4\%}$	$Ps_{2\%}P_{4\%}$	$(DMD)_{2\%}P_{4\%}$
Brightness	54.2	46.8	45.7	54.7	56.0	57.2
Yield %	93.0	99.5	99.8	93.8	94.7	96.4

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Mechanistic Aspects of Bleaching Chemistry

In this part, relevant bleaching mechanisms are reviewed and the results of new model compound studies are discussed in relation to the observations of pulp bleaching.

Coniferaldehyde- and stilbene-type structures are well documented as chromophores or leucochromophores present in wood and/or formed during pulping (*25*, *26*, *27*, *28*). Destruction of these chromophores is important for brightness gain in the mechanical pulp bleaching (*1*, *6*). In our earlier work (*29*, *30*), the reactivities of the related model compounds towards hydrogen peroxide and peracetic acid were compared. Overall, peracetic acid reacted with all the model compounds to a greater extent and at a faster speed than hydrogen peroxide. There was also a difference between these two bleaching agents regarding reaction pathways. For instance, the reaction of coniferaldehyde with peracetic acid selectively gave a colorless product that did not seem to undergo secondary reactions and the undesirable consumption of the oxidant.

However, the peracetic acid advantage observed in the lignin model compound study is not translated into a more effective pulp bleaching. As found earlier, peracetic acid is less effective in bleaching softwood and hardwood mechanical pulps than hydrogen peroxide on a weight basis of bleach chemical charge. There is no synergy between hydrogen peroxide, peracetic acid and peroxymonosulfate. Further fundamental study is needed to understand more fully the origin of the brightness ceiling of mechanical pulp bleaching.

In contrast to the wood pulps, wheat straw pulp can be more efficiently bleached by combining peracids with hydrogen peroxide. Wheat straw may contain an appreciable amount of non-lignin coloring substances. Ferulic acid is one of them. Our earlier study has demonstrated that ferulic acid and its derivatives are fairly unreactive with hydrogen peroxide and undergo bond cleavage only to a small extent with peracetic acid (*17*). Under the identical treatment, coniferaldehyde model compounds are almost completely degraded. As a continuation of our endeavors in fundamental research in this field, the reactivity of model compounds related to ferulic acid, as illustrated in Figure 8, toward peroxymonosulfate and DMD is reported in the present work.

Table V shows that ferulic acid is quite reactive to peroxymonosulfate. In the presence of acetone, the *in situ* generated DMD results in a complete consumption of the substrate. The esterified or etherified derivative shows a similar reactivity as compared to ferulic acid. Nevertheless, diferulic acid appears to be slightly less reactive, likely due to the availability of only half of the amount of active oxygen per ferulic acid unit for the monomeric model compounds. While the extent to which the ferulic acid-based structures contribute to the color of wheat straw is yet to be understood, the results obtained here can serve to explain the enhancement of brightness response of

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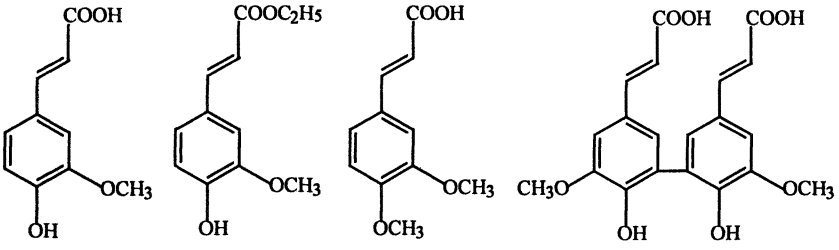
wheat straw pulp by the new bleaching reagents. It is evident that certain peroxide-resistant structures can be degraded by peroxymonosulfate and DMD.



I II III IV

Figure 8. Model compounds studied: ferulic acid (I), ethyl ferulate (II), 3,4-dimethoxycinnamic acid (III), and 5, 5'-diferulic acid (IV)

Table V. Reactivity of Model Compounds with Peroxymonosulfate (Ps) or *in situ* Generated DMD at room temperature (22 °C) for 3 h

Reaction	*Substrate Consumption (%)*
Ferulic acid with Ps	63.3
Ferulic acid with DMD	97.2
Ethyl ferulate with DMD	96.5
3,4-Dimethoxycinnamic acid with DMD	98.0
Diferulic acid with DMD	88.7

Conclusions

Peracetic acid and peroxymonosulfate can be alternatives to hydrogen peroxide in mechanical pulp bleaching. As stronger oxidizing reagents, the peracids are capable of attacking peroxide-resistant chromophores, thereby resulting in enhanced bleaching effect. In contrast to the hydrogen peroxide bleaching that requires high alkalinity, the peracid bleaching is carried out under neutral or mildly alkaline conditions. Such a moderate treatment minimizes the dissolution of lignin and hemicelluloses in bleaching. Consequently, the pulp yield loss in peracid bleaching is considerably smaller than that caused by hydrogen peroxide. Nevertheless, the benefits of peracid replacement for hydrogen peroxide depend upon the species of pulps and, particularly, the nature of their chromophores.

The benefit of additional brightness gain by the peracids is not evident in bleaching wood pulps. It seems likely that hydrogen peroxide and the peracids react with the wood chromophores in a similar manner. Thus, no synergy is observed when these two types of oxidants are employed in combination. In fact, DMD is such a very powerful oxidant and it has an adverse effect on the brightness development of wood pulps. Apparently, yield conservation is the main benefit from bleaching wood mechanical pulps with the peracids. Partial replacement of hydrogen peroxide by a peracid allows a reduction in the alkalinity required to achieve the target brightness. More pulp components, particularly hemicelluloses, are retained.

In addition to the pulp yield benefit as described above, the bleaching enhancement is a major benefit from bleaching wheat straw pulp with peracids. As wheat straw contains a substantial amount of peroxide-resistant chromophores, using the peracids or the even stronger DMD is found to be very beneficial to the bleaching of wheat straw.

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Chapter 10

Intrastage Recycling of Effluents from Activated Peroxide, Peracetic Acid, and Ozone Bleaching

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Reducing the quantity of fresh water in a bleach plant can cause an accumulation of dissolved organic material and non-process elements in the recycled filtrates and partly on the fiber surface. The effect of bleaching filtrate recycling within each stage and the significance of various metals and chelating agents for efficiency and selectivity in ozone, peracetic acid, and molybdate activated acidic peroxide delignification were studied. The results indicate that when these delignification filtrates are recycled, metals accumulate in the filtrates and fibers. Manganese and DTPA in combination catalyzed the degradation of peracetic acid. The degradation reaction mechanism will be discussed in this chapter. The chelating agent DEAS, which has a low nitrogen content, stabilized peracetic acid in the presence of manganese. The oxalate content in bleaching filtrates of peracetic acid and peroxomolybdate (mP) was low compared with a corresponding filtrate from the ozone stage. The mP filtrate mostly contained formic acid and the filtrate from the peracetic acid stage was rich in acetic acid. Peroxide consumption did not increase in the mP stage since the reaction products with a low molecular weight do not consume much peroxide under acidic conditions.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Introduction

In modern pulp mills, environmental concerns have led to a reduction in the amount of fresh water used. Because the bleach plant is the open part of the pulp mill, recycling of bleaching filtrates within each stage has become increasingly important. Modified cooking methods and new bleaching technologies have resulted in lower amount of harmful components in bleaching filtrates. Consequently, the risk of an accumulation of dissolved organic and inorganic material on the fibers and in the filtrate has increased. This can lead to a wasteful consumption of bleaching chemicals and scaling problems in the bleach plant, due to precipitation of metal salts such as calcium oxalate. System closure also increases the concentration of elements originating from wood, such as Al, Si, P, Cl, K, Ca and Ba. This can cause problems in the operation of the chemical recovery system, e.g. pluggings of the recovery boiler, scaling in the evaporators, and corrosion of the chemical recovery equipment (*1,2,3*).

Different processes have been published as alternative methods for removing organic and inorganic matter from bleaching filtrates. These methods include membrane filtration (*1,4*), ion exchange technology (*1*), and coagulation and flocculation (5). A modification of the latter alternative is used as industrial TCF bleach plants to remove harmful metals in the chelation-stage filtrate. It also allows recovery of chelating agent leading to less use of fresh water and bleaching chemicals (*6*).

As mentioned earlier, calcium oxalate can be a problem when bleaching filtrates are recirculated. Oxalic acid is formed when lignin and hexenuronic acids (HexA) react with acidic oxidative bleaching chemicals, e.g. chlorine dioxide and ozone. During kraft cooking HexA-groups are formed from the 4-O-methylglucuronic acid groups in xylan. Lignin and HexA-groups consume potassium permanganate in kappa number determination, i.e. HexA-groups are part of the kappa number determined (*7*). In the presence of calcium ions and oxalic acid the risk of calcium oxalate precipitate increases when an alkaline bleach filtrate is mixed with the acidic filtrate. Hexenuronic acids in pulp can also be removed without any bleaching chemicals, by using acid hydrolysis (*7,8*). The loss of bleached pulp strength and viscosity has been reported to be more detrimental when acidic hydrolysis is used instead of oxidation of hexenuronic acids (*8*). The HexA-groups in pulp also bind transition metals, which can be removed from pulp more easily during and after HexA removal (*7*).

In ozone delignification, the formation of non-selective radicals and alkali-labile carbonyl groups, and the quantity of dissolved carbohydrates and oxalic acid is dependent on the ozone charge (*9,10*). Radicals such as superoxide and hydroxyl are formed in reactions between ozone and lignin structures (*9,11,12*). It has been reported that ozone does not react directly with carbohydrates, but carbohydrate degradation occurs if a high amount of ozone is present in order to

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produce radicals from lignin (*11,12*). Transition metals have been found to be of minor importance in producing radicals, due to the fast reaction between ozone and organic material (*13*). In ozone delignification oxalic acid is formed from HexA-groups, but residual lignin can also be a source of oxalic acid when high ozone charges are used (*8,9*). A major part of the oxalic acid formed during ozonation has been reported to be liberated during the subsequent Q-stage (*14*).

Unlike ozone, during peracetic acid (PAA) delignification only a very small amount of oxalic acid is formed. Formic acid is formed as a reaction product in reactions between PAA and HexA-groups (*8,15,16*). PAA also seems to reduce the amount of alkali-labile carbonyl groups in pulp, thus preventing carbohydrates from degrading and dissolving in the subsequent alkaline bleaching stage (*9,10*). During PAA delignification the quantity of dissolved carbohydrates is also minor over a pH range of 4 to 7 (*15*). PAA may also oxidize alkali-labile carbonyl groups in pulp formed during ozone delignification to carboxyl groups (*10*).

A peracid-type chemical which can remove hexenuronic acids can be formed by adding molybdate to a pulp slurry where peroxide is present under weakly acidic conditions. A suitable pH level is <5. A pH lower than 3.5 results in a high degree of delignification, but degradation of carbohydrates takes place. At a pH higher than 6, molybdate degrades H_2O_2 rapidly and no significant delignification occurs *(17)*. The peroxomolybdate that is formed oxidizes the HexA-groups forming formic acid (*8,18*). The build-up of oxalic acid in peroxomolybdate delignification (mP) is minor. Peroxomolybdate also removes lignin with high selectivity, as well as removing extractives from the pulp (*17,19*). Peroxomolybdate has been considered attractive because Mo can be recycled and the catalyst charge needed would therefore be lower. The dissolved organic compounds are mostly organic acids of low molecular weight that can be recycled with molybdate without any additional consumption of peroxide. The recycling also reduces the bleaching costs, since Mo is relatively expensive. Thanks to the efficient removal of HexA, the formation of oxalate in the subsequent bleaching stages is reduced (*18*). Various types of molybdates can be utilized as a catalyst, heteropolymolybdates being especially suitable (8,*11,26*). In mill scale applications the make-up level of Mo is around 50-100 g/ton of pulp. However, the major problem with the use of peroxomolybdate has been reported to be recovery of the catalyst (*18*). Mo does not have a negative impact on the recovery cycle or kraft cooking. In wastewater treatment, Mo residuals have been shown not to have a negative effect on either the microbes or the amount of precipitate after sedimentation.

In TCF and ECF_{light} bleaching, chelating agents such as EDTA and DTPA are used to control the metal profile. Any excess of EDTA or DTPA has been reported to degrade in the presence of high charges of ozone, peracetic acid or chlorine dioxide (*20,21*). While DTPA inhibits the negative effects of iron in PAA-delignification, the Mn-DTPA or Mn-EDTA complex formed catalyzes the

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decomposition of peracetic acid (*16,25*). It has been proposed that this is due to the unstable DTPA-Mn(II) complex and the participation of Mn in the oxidation-reduction cycle (*22*). Greater decomposition of bleaching chemicals and chelating agents can occur if filtrates containing chelating agents are recycled within the bleach plant back to a previous bleaching stage which contains a strong oxidative bleaching chemical.

The purpose of this chapter is to discuss how a higher circulation of bleaching filtrates influences bleaching chemical consumption, pulp quality, chemical balance and the amount of fresh water needed. Bleaching filtrates are analyzed and the importance of different organic and inorganic materials is discussed.

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Experimental

Northern European HW birch kraft pulps were used as the raw material in the laboratory experiments. The pulp properties were determined according to SCAN standards. The determination of hexenuronic acids (HexA) in the pulp was based on the selective hydrolysis and spectrophotometric determination of the furan carboxylic acid formed (7). Distilled 38% PAA was used in the bleaching experiments. The distilled product contained free AA (< 4%) and H_2O_2 (< 2%). The chelating agent dosages were calculated on the basis of 100% sodium salts. DEAS was prepared via a lanthanide-catalyzed Michael addition of diethanolamine to maleic acid sodium salt (27). The oxalates were measured as dissolved and total oxalate. The dissolved oxalate was determined from a filtered sample by ion chromatography. The total oxalate was determined by acidifying the sample to pH 2 prior to filtering.

Bleaching filtrate recycling tests were carried out as shown in Figure 1. The effluent was pressed out from the pulp slurry after bleaching. This pressate was used as dilution water for the second round of bleaching. The second-round pressate was added in the third round test, and so on. Altogether five circulation rounds were performed using ozone, peracetic acid and peroxomolybdate separately. In every bleaching test the fraction of the effluent from the previous stage was 66% of the total quantity of water used. The degree of closure in these experiments was high in order to demonstrate the effect of build-up of harmful substances during intra-stage recirculation. The chelating agent dosage was initially 1 kg/tp and in the following cycles 0.5 kg/tp. The chelating agent dosage was selected according to the initial metal levels in the pulp, the reactions between the chelating agents and bleaching chemicals, and the amount of recirculated chelating agent.

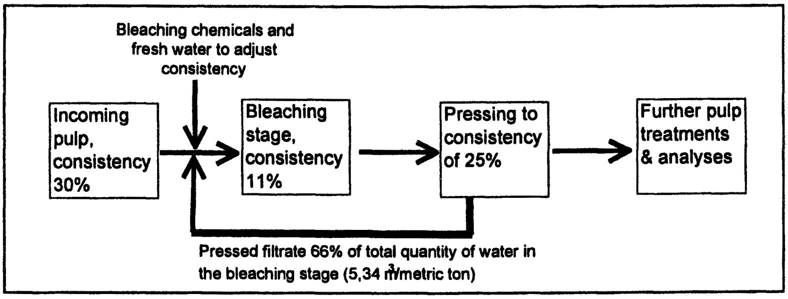
Bleaching chemicals and fresh water to adjust consistency
Incoming pulp, consistency 30%
Bleaching stage, consistency 11%
Pressing to consistency of 25%
Further pulp treatments & analyses
Pressed filtrate 66% of total quantity of water in the bleaching stage (5,34 m³/metric ton)

Figure 1. Schematic presentation of the procedure used in the recycling tests.

The reaction conditions in bleaching trials were as follows:
Filtrate recycling tests, i.e. Figures 6 and 7 and Table 1: O-Q-OP prebleached HW kraft pulp, initial kappa 6.6, HexA 40 mmol/kg. PAA/Q: 60/30min, 80°C, Cs 11%, pH 6, PAA 5 kg/tp. Z/Q: 2/30 min, 60°C, Cs 11%, pH 3.5/5.5, O_3 3 kg/tp. mP/Q 120/30min, 80°C, Cs 11%, pH 5/6, Mo 0.4 kg/tp, H_2O_2 5 kg/tp. There was no intermediate washing between the PAA (Z, mP) and Q stages, merely addition of chelating agent and adjustment of pH (NaOH). The final bleaching was carried out with alkaline peroxide, Cs 11%, 200 min., 90°C, initial pH 10.5, H_2O_2 15 kg/tp, $MgSO_4$ 1 kg/tp. The pulps were 2 x washed with deionized water (80°C) prior to final bleaching with peroxide. The water/oven-dry pulp ratio in washing was 20/1.

Figures 8 and 9: Oxygen-delignified HW kraft pulp with a kappa number of 10.4 (HexA 36 mmol/kg). The degree of delignification of the PAA/Q, Z/Q and mP/Q stages was about 4 kappa units, a sample of the filtrate being taken at the reaction temperature. PAA/Q: 120 min, 80°C, pH 6, PAA 12 kg/ton pulp, Cs 10%, subsequent chelation (without intermediate washing) with 2 kg/tp DTPA. Z/Q: 4 min, 50°C, pH 3, O_3 4 kg/ton pulp, Cs 10%, subsequent neutralization without intermediate washing, 30 min, 50°C, Cs 9%, pH 6.5, 2 kg/tp DTPA. mP/Q: 120 min, 80°C, pH 5, H_2O_2 8 kg/ton pulp, Mo 0.4 kg/tp, Cs 10%, subsequent chelation (without intermediate washing) with 2 kg/tp DTPA, pH 6.5. The chemical dosages were calculated as 100% active substance per oven-dry metric ton of pulp.

Results and Discussion

As is well known, wood is the main source of most metals in kraft pulp and in bleaching filtrates. In particular, the metals that can cause problems in the

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bleaching plant originate mainly from wood. These metals include calcium, manganese and iron. Figure 2 shows metal levels of several Finnish pulp mills.

As can be seen from Figure 2, the pulp usually contains only minor amounts of copper (average about 0.5 ppm). However copper can be a problem in the case of laboratory experiments when alkaline kraft pulps are washed with tap water (*23*). Some of the magnesium content in the pulps may originate from the $MgSO_4$ added in oxygen delignification. The variation in metal levels in pulp mainly derives from the location in which the wood grew. The wood harvesting season and process waters also influence metal levels. In TCF and ECF_{light} bleaching, the varied metal level influences the metal management and the need for chelating agents.

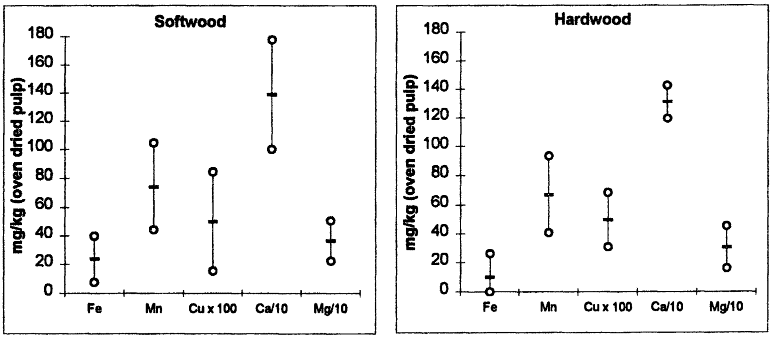

Figure 2. Metal contents of oxygen-delignified SW and HW (birch) kraft pulps. 40 samples from 5 pulp mills in central and northern Finland. Samples were taken at all seasons of the year.

In order to reduce fresh water consumption, the filtrates should be recycled at least to some extent. A typical solution for filtrate circulation is shown in Figure 3.

In the process presented in Figure 3, some of the filtrate is discharged into the effluent treatment process or some other previous bleaching stage. Some of the filtrate is recycled back to the same bleaching stage, where some fresh water or washing water from a subsequent bleaching stage is also used on the showers of the washers. However, in this kind of water recirculation system, dissolved organic material, metals and the chelating agent are also circulated. This can lead to an accumulation of organic material and metals, and higher bleaching chemical consumption. Furthermore, the chelating agent can also be decomposed in reactions with ozone, peracetic acid or chlorine dioxide. The build-up of

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harmful substances in the filtrate also increases the washing losses and may thus raise chemical consumption and decrease pulp quality in the following bleaching stages.

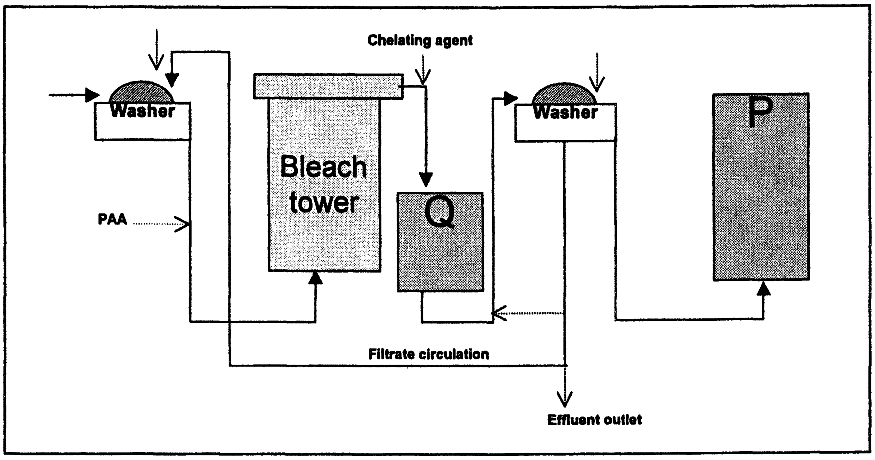

Figure 3. A possible scheme for bleaching filtrate recycling in peracetic acid or ozone bleaching combined with subsequent chelation.

A stability test on peracetic acid in the presence of DTPA, EDTA as well as manganese and iron showed that peracetic acid rapidly decomposes in the presence of DTPA and manganese (see Figure 4).

Manganese or iron alone does not decompose peracetic acid as rapidly. A high amount of DTPA alone consumes some peracetic acid, but the catalytic effect of Mn-DTPA is much more rapid and detrimental. DTPA in the absence of Mn consumes about 3 moles of PAA per mole of DTPA, which corresponds well with the earlier observation (*22*). This can be attributed to oxidation of tertiary nitrogens in the DTPA molecule to N-oxides *(29)*. We assume that the mechanism of rapid decomposition of PAA in the presence of manganese and DTPA is due to the active Mn-complex of DTPA-N-oxide. Peroxo-ligands formed as intermediates are linked to the manganese center of the oxidized Mn-DTPA complex and participate in an oxidation-reduction cycle of manganese, causing rapid decomposition of PAA. In the presence of peracetic acid, this catalytic decomposition mechanism may take place in the case of metal complexes, where the nitrogens of the ligand are coordinated with the central atom of the metal complex. In the case of iron complexes, this rapid degradation does not occur, presumably because of the higher stability of Fe-DTPA and Fe–

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EDTA complexes, i.e. iron does not participate in an oxidation-reduction cycle. This is also valid in the case of copper complexes.

Test	mg/l PAA	Chelating agent	Dose mg/l	Mn mg/l	Fe mg/l	mmol/l PAA	Chelant mmol/l	PAA:L Molar ratio
A	1000	None	0	0	0	13,2	0,0	-
B	1000	DTPA	100	0	0	13,2	0,2	66,2
C	1000	DTPA	1000	0	0	13,2	2,0	6,6
D	1000	EDTA	1000	0	0	13,2	2,6	5,0
E	1000	None	0	0,5	0	13,2	0,0	-
F	1000	None	0	0	0,5	13,2	0,0	-
G	1000	DTPA	100	0,5	0	13,2	0,2	66,2
H	1000	DTPA	100	0	0,5	13,2	0,2	66,2
I	1000	DEAS	100	0,5	0	13,2	0,2	73,3

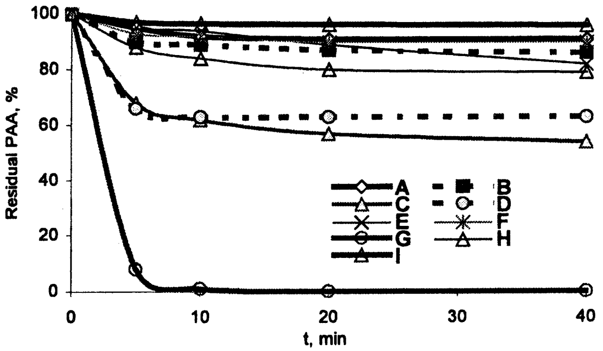

Figure 4. The effect of chelating agent, manganese and iron on the amount of residual peracetic acid in de-ionized water at pH 5.5 and 60°C. The chelating agent and metal were mixed 10 min before the PAA was added. The experiment started when the PAA was added (0 min).

Some nitrogen-containing chelating agents, like DEAS, do not decompose peracetic acid in the presence of manganese, however. The chemical structures of DTPA and DEAS are shown in Figure 5. DEAS is a complexing agent with low nitrogen content that, according to reference *24*, does not decompose peracetic acid and is more stable in oxidizing conditions, but is not yet available for commercial distribution.

The result of these stability tests in Figure 4 shows that the behavior of oxygen-based chemicals in the presence of different metal-chelating agent complexes is not sufficiently understood. The detrimental effect of Mn-DTPA in

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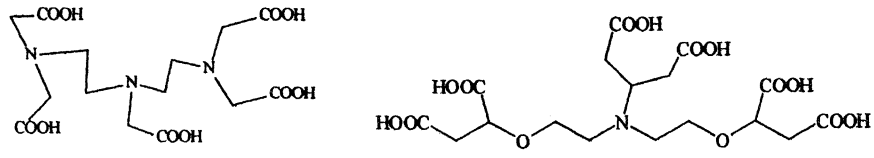
PAA delignification depends on the kappa number of the pulp. Pulps with a residual kappa number above 8 have shown some stabilizing behavior to counter this catalytic effect. The presence of magnesium has also been shown to have a PAA protective influence with respect to manganese in the presence or absence of DTPA or EDTA. Minimizing the amount of DTPA or EDTA also results in more calcium remaining in the pulp.



$C_{14}H_{23}N_3O_{10}$, N 10.7%

DTPA

$C_{17}H_{25}NO_{14}$, N 3.0%

DEAS

Figure 5. Chemical structures of DTPA and DEAS (N-bis[1,2-dicarboxy-ethoxy)-ethyl]-aspartic acid).

Figure 6 demonstrates the effect of bleaching effluent recycling within the bleach plant using a PAA/Z/mP sequence with different chelating agents (EDTA, DEAS). The procedure used in these experiments is presented in Figures 1 and 3.

The results in Figures 6 and 7 show that peracetic acid will be wasted at advanced levels of filtrate recycle due to the accumulation of manganese in the presence of EDTA. In ozone delignification kappa number reduction declined slightly after the first round of recirculation. This was due to dissolved organic matter that consumed some of the bleaching chemical. The lowest pulp viscosity losses during recirculation were obtained in mP/Q-P bleaching. The highest relative viscosity losses during filtrate circulation were obtained in PAA/Q(DTPA)-P and Z/Q-P bleaching (Figure 6). The circulation increased peroxide consumption in the subsequent alkaline peroxide stage less than 1.5 kg/tp in all cases. The final brightness for O-Q-OP-PAA/Q(DEAS)-P, O-Q-OP-Z/Q-P and O-Q-OP-mP/Q-P bleached pulps was around 89% ISO in every case. Peroxide consumption increased somewhat and pulp viscosity deteriorated as filtrate circulation increased. DEAS effectively stabilized PAA-delignification compared with EDTA. DEAS is more biodegradable than EDTA or DTPA, but is stable against degradation under filtrate recirculation conditions.

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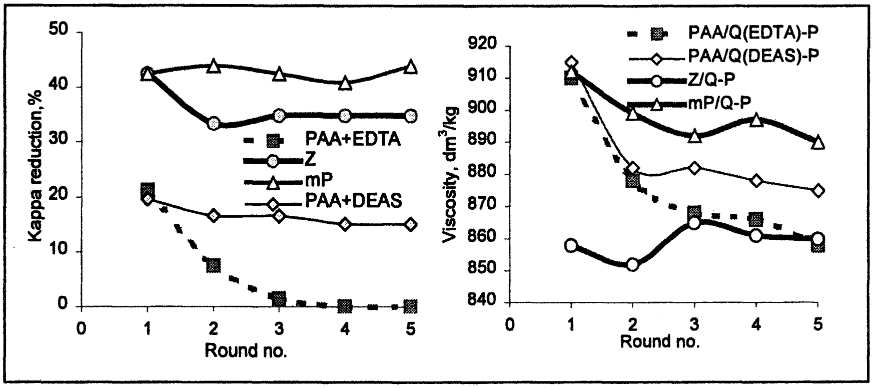

Figure 6. Kappa reduction (left) and pulp viscosity after final bleaching with alkaline peroxide (right) as a function of the degree of filtrate recirculation.

The corresponding HexA content of HW kraft pulps after the various delignification treatments is shown in Figure 7.

As can be seen from Figure 7, mP is most efficient in removing HexA, as filtrate recirculation does not affect the removal of HexA. Since the reaction products in mP filtrate are mainly organic compounds with low molecular weight, they do not significantly consume chemicals when the filtrate is recirculated. In the presence of EDTA, peracetic acid is degraded and thus the removal of HexA is negligible. The accumulation of metals in the pulp and filtrate is shown in Table 1.

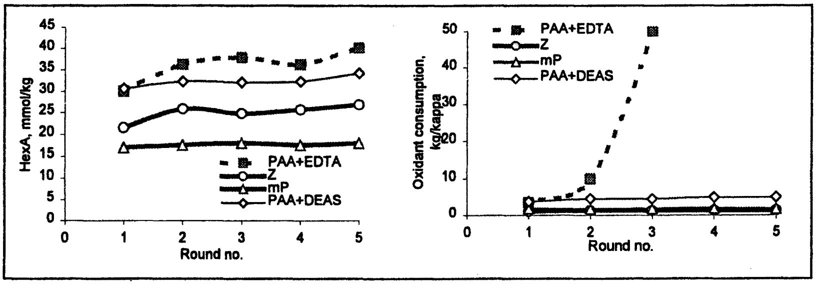

Figure 7. Residual HexA content in pulp (left) and chemical consumption per kappa unit (right) as a function of the degree of filtrate recirculation.

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Table I. Accumulation of metals in pulp and filtrate during the bleaching trials in Figures 6-7. Initial metal content of the pulp: Al 12 mg/kg, Ca 680 mg/kg, Cu <0.5 mg/kg, Mg 380 mg/kg, Mn 4.7 mg/kg, Fe 5.2 mg/kg. The pulp had already been chelated once at the mill.

Untreated pulp					
Fe	5.2	*ppm*	*Al*	12	*ppm*
Mn	4.7	*ppm*	*Mg*	380	*ppm*
Ca	680	*ppm*	*Cu*	<0.5	*ppm*

	PAA/Q (EDTA)							
	Pulp				Filtrate			
Round no.	*Fe, ppm*	*Mn, ppm*	*Mg, ppm*	*Ca, ppm*	*Fe, ppm*	*Mn, ppm*	*Mg, ppm*	*Ca, ppm*
1	2,9	0,5	290	490	<0,5	0,7	16	32
2	3,0	0,7	305	510	0,6	1,1	24	42
3	3,0	1,1	325	565	0,6	1,5	30	48
4	3,1	1,4	343	603	0,7	1,6	32	49
5	3,1	1,7	360	640	0,7	1,6	33	48
	Z/Q							
	Pulp				Filtrate			
Round no.	*Fe, ppm*	*Mn, ppm*	*Mg, ppm*	*Ca, ppm*	*Fe, ppm*	*Mn, ppm*	*Mg, ppm*	*Ca, ppm*
1	4,9	0,9	210	420	0,6	0,7	32	53
2	4,0	1,0	215	440	0,8	1,3	54	79
3	4,3	1,4	240	470	1,0	1,7	74	100
4	3,9	1,6	260	495	1,0	2,0	88	120
5	3,6	1,8	270	520	1,1	2,1	93	120
	mP/Q							
	Pulp				Filtrate			
Round no.	*Fe, ppm*	*Mn, ppm*	*Mg, ppm*	*Ca, ppm*	*Fe, ppm*	*Mn, ppm*	*Mg, ppm*	*Ca, ppm*
1	2,9	0,9	285	475	0,7	0,7	18	36
2	2,9	1,0	299	498	1,0	1,2	22	44
3	3,1	1,3	324	556	1,1	1,9	31	46
4	3,1	1,5	343	596	1,2	2,1	33	47
5	3,2	1,7	362	636	1,2	2,1	33	50

Despite the filtrate recirculation, the metal content in the pulp remains relatively low. Due to the lower reaction pH, ozone delignification dissolved more alkaline earth metals, especially calcium. Calcium can cause deposits with oxalate and fatty acids originating from extractives.

Figures 8 and 9 present the relative oxalate and formic acid contents in bleaching filtrates after the PAA/Q, mP/Q and Z/Q stages.

The acetic acid in PAA filtrate mostly originates from peracetic acid. The oxalate content of PAA and mP filtrate is low compared with ozone filtrate.

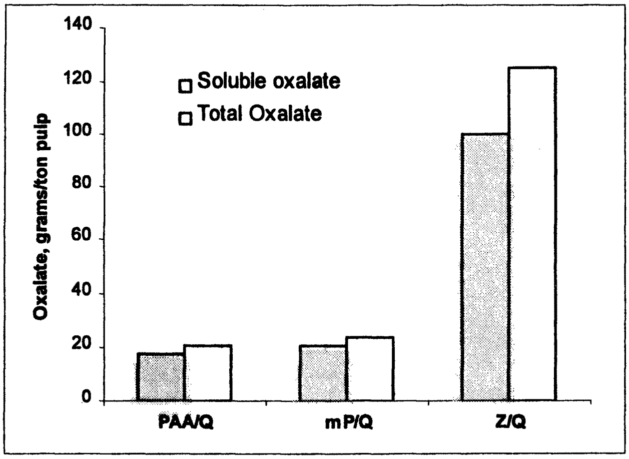

Figure 8. Formation of oxalate at different bleaching stages. The HW kraft pulp used was delignified by about 4 kappa units in a single stage.

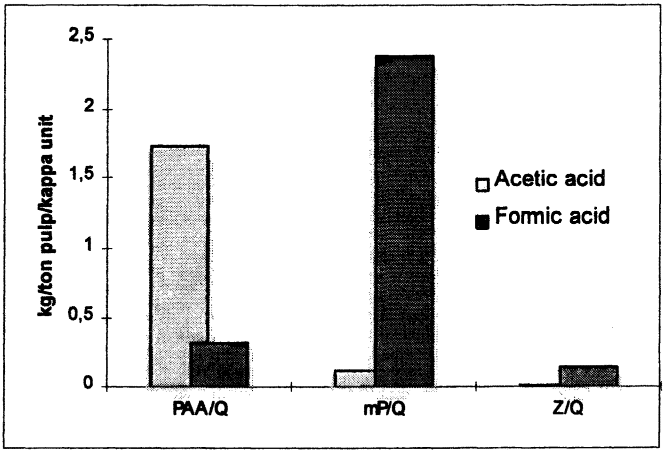

Figure 9. Acetic acid (left) and formic acid (right) in filtrates from different bleaching stages.

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The active species in molybdate-activated peroxide delignification is the peroxomolybdate that is formed in the reaction between H_2O_2 and molybdate. After the peroxomolybdate has reacted with oxidizable species, the remaining molybdate can react with H_2O_2 again. The assumed reaction mechanism of peroxomolybdate with HexA involves an epoxidation reaction of a double bond followed by an epoxide ring opening. The diol structure formed is then hydrolyzed and the decomposition products formed are further oxidized by peroxomolybdate. The proposed pathway of HexA's reaction to formic acid is shown in Figure 10.

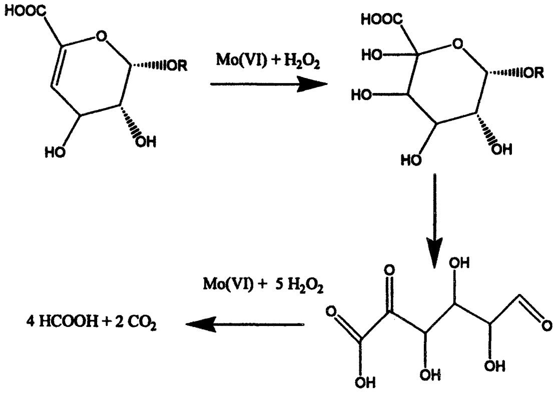

Figure 10. Proposed reaction of HexA with molybdate-catalyzed peroxide.

However, it should be noted that HexA is not necessarily the only origin of formic acid. Formic acid is also formed in reactions with lignin. According to studies carried out by Sundman (28), the main reaction mechanisms of peroxomolybdate with lignin involve demethoxylation and the formation of quinoid structures, though the peroxomolybdate was not a strong enough oxidant to facilitate ring rupture in most cases, and both the side-chain in apocynol and the olefinic bonds were attacked.

One kappa unit corresponds to 10 units of HexA (7), and if HexA consumes H_2O_2 in a molar ratio of 6:1, the peroxide consumption in the mP stage should be about 2 kg H_2O_2/ton of pulp per kappa unit. In practice, peroxide consumption has been reported to be slightly lower, about 1.5 kg H_2O_2/ton of pulp per kappa unit (18). However, even if mP removes HexA efficiently, only part of the kappa reduction in the mP stage is due to HexA. In these filtrate recirculation experiments, the consumption of H_2O_2 in the mP stage was about 1.8-2 kg H_2O_2/ton of pulp per kappa unit.

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Conclusions

DTPA and EDTA degrade peracetic acid, especially in the presence of manganese. DEAS, which has a low nitrogen content, stabilized PAA even in the presence of Mn. When peracetic acid is used as a delignifying agent in existing bleach plants with low fresh water consumption, the effects of different chelating agents should be taken into account.

When filtrates of PAA, Z and mP stages are recirculated, transition metals accumulate on the fibers and in the filtrates. However, in peracetic acid bleaching the detrimental effect of transition metals is minor compared with the catalytic degradation of PAA caused by manganese complexes of EDTA and DTPA. The amount of EDTA or DTPA used in bleaching processes should be minimized to avoid higher consumption of bleaching chemical.

The filtrates from an ozone stage contained high levels of oxalate. Furthermore, since the recirculation of filtrates leads to an accumulation of dissolved calcium, there is a change that deposits may form. The precipitation of Ca-oxalate is most likely in places where the pulp pH is changing from alkaline to acidic or vice versa, i.e. in washers.

Filtrate recirculation did not increase peroxide consumption in a molybdate-activated peroxide stage. The mP filtrates contained only a minor amount of oxalate. Since filtrate circulation seems to have no significant adverse effect on delignification efficacy, there seem to be no technical restrictions in this respect in apply this method at existing bleach plants.

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Chapter 11

Oxidative Degradation of Dimeric Lignin Model Compounds

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The oxidation of dimeric lignin model compounds (arylglycerol - β - aryl ethers and phenylcoumarans) by molecular oxygen, catalyzed by [N, N′ - bis(salicylidene) - ethane - 1, 2 - diaminato] cobalt(II), [Co(salen)], was studied in order to find novel processes for the production of chemicals by degradation of polyphenols contained in waste waters from the pulp and paper industry.

All the studied compounds reacted at room temperature in chloroform with high conversion already after 30 minutes; after 48 h the reaction was essentially complete and interesting low molecular weight compounds were isolated with good yields.

An EPR investigation of the reaction mixtures indicates the presence of a phenoxy cobalt radical, [$\mathrm{Co^{III}(salen)(RO^-)(RO^\bullet)}$], as a reactive intermediate for ROH as phenolic substrate. The electron donor properties of the phenolic substrate and the stability of the phenoxy radical control the yields of the oxidation products.

Introduction

The use of renewable biomass as raw material for the production of chemicals is an important component of international energy policy. One of the most underused carbon source materials in the biosphere is lignin, present in the woody structure of higher plants, typically 30% in dry weight (*1*). It is a structurally highly intricate aromatic polymer of oxygenated phenylpropane units (*2*, *3*, *4*, *5*, *6*). Several interunit carbon-carbon and carbon-oxygen bonds are present in its structure, the relative abundance of these interunit linkages varying for the different types of wood. Many studies are focused on their oxidative degradation to give useful low molecular weight aromatic compounds such as vanillin. These processes are commercially important and may be performed by oxidation with nitroaromatics (*7*), with air in alkaline solution (*8*), with ozone (*9*) and by electrochemical means (*10*). Other degradation systems use dioxygen as the oxidant and cobalt-complexes as catalysts. Drago et al. showed that dioxygen was able to oxidize, in very good yield, isoeugenol to vanillin, using [bis(salicylidene-γ-iminopropyl)methylamine]cobalt(II), [Co(SMDPT)], as catalyst (*11*); recently Bozell et al. reported the oxidation of *para*-substituted phenolic compounds to benzoquinones using [N,N′-bis(salicylidene)-ethane-1, 2-diaminato]cobalt(II), [Co(salen)], as catalyst (*12*, *13*).

We have recently reported that simple model compounds of the polyphenols contained in the waste waters from the paper industry and agroindustrial activity can be degraded by oxidation with dioxygen, using [Co(salen)] as catalyst (*14*). The mechanism of the reaction was elucidated by studying the degradation of several phenolic propenoids with different electron donor properties and by monitoring the process by electron paramagnetic resonance (EPR) spectroscopy (*15*). Among the investigated compounds, E-methyl ferulate (compound **1** in Scheme 1) has higher electron donor capacity than methyl E-4-hydroxycinnamate (compound **2** in Scheme 1).

Structures like **1** and **2** are very simple model compounds for lignin, containing only one aromatic ring with an extended double bond-ester conjugation (*16*). However, since only a very small amount of such structures are present in lignin waste material, we decided to test model compounds representing more abundant structural units (*17*) as substrates of oxidation.

Arylglycerol-β-aryl ethers are the most abundant structural units of lignin (*17*), phenylcoumarans are also abundant and contain an intermonomeric carbon-carbon bond (C_8-C_5) (*18*). The model compounds with two aromatic rings are indicated as "dimeric" lignin model compounds, to distinguish them from phenols containing one ring, indicated as "monomeric" model compounds.

Thus, the oxidation of arylglycerol-β-aryl ethers (compounds **3**, **4**, **5** in Scheme 2) and phenylcoumarans (compound **6** in Scheme 3) by molecular oxygen, catalyzed by [Co(salen)], was studied with the aim of obtaining further insight into the oxidation mechanism of lignin model compounds.

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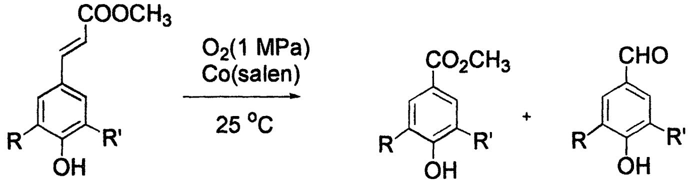
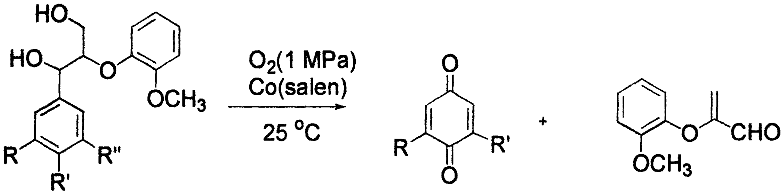

COOCH$_3$
R
R'
OH
O$_2$(1 MPa)
Co(salen)
25 °C
CO$_2$CH$_3$
R
R'
OH
+
CHO
R
R'
OH

1 R = H; R' = OCH$_3$
2 R = R' = H

7 R = H; R' = OCH$_3$
9 R = R' = H

8 R = H; R' = OCH$_3$
10 R = R' = H

Scheme 1: Monomeric Compounds

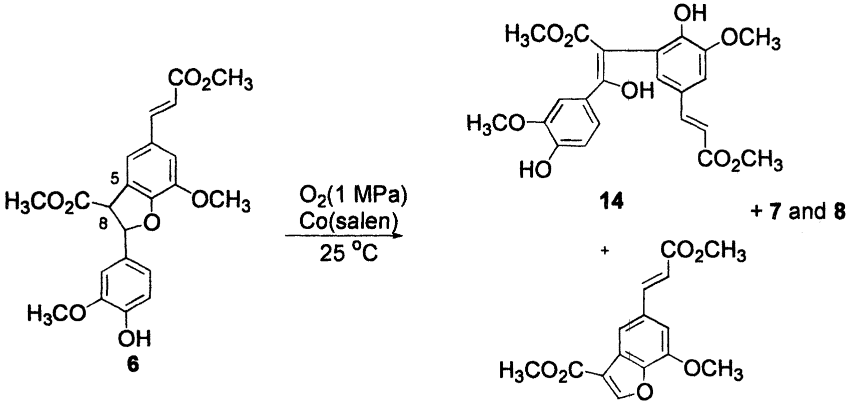

HO
HO
O
OCH$_3$
R
R''
R'
O$_2$(1 MPa)
Co(salen)
25 °C
O
R
R'
O
+
O
CHO
OCH$_3$

3 R = OCH$_3$; R' = OH; R'' = H
4 R = R'' = H; R' = OH
5 R = R'' = H; R' = OCH3

11 R = OCH$_3$; R' = H
13 R = R' = H

12

Scheme 2: Dimeric Compounds


CO$_2$CH$_3$
5
H$_3$CO$_2$C
8
O
OCH$_3$
H$_3$CO
OH
6
O$_2$(1 MPa)
Co(salen)
25 °C
OH
H$_3$CO$_2$C
OCH$_3$
OH
H$_3$CO
HO
CO$_2$CH$_3$
14
+ 7 and 8
+
CO$_2$CH$_3$
H$_3$CO$_2$C
OCH$_3$
O

15

Scheme 3: Dimeric Compounds

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The reactivity was measured as conversion (disappearance of starting material) and the product distribution was determined after methylation using GC-MS. The reactions were monitored by EPR spectroscopy.

Experimental

Reagents

The complex [Co(salen)] (99%) and the 1, 4 - benzoquinone, used as standard, were from Aldrich. Chloroform (Fluka) was used as received.

The dimeric substrates 3 - (4 - hydroxy - 3 - methoxyphenyl) - 2 - (2 - methoxyphenoxy) - 1, 3 - propanediol **3**; 3 - (4 - hydroxyphenyl) - 2 - (2 - methoxyphenoxy) - 1, 3 -propanediol **4**; 3 - (4 - methoxyphenyl) - 2 - (2 - methoxyphenoxy) - 1, 3 - propanediol **5** were prepared according to the method of Sipila and Syrjanen (*19*). E - methyl - [(2RS - 3SR) - 2, 3 - dihydro - 2 - (4 - hydroxy - 3 - methoxyphenyl) - 7 - methoxy - 3 - methoxycarbonyl - 1 - benzofuran - 5 - yl] propenoate **6** was prepared according to the method of Bolzacchini et al. (*20*).

Apparatus

NMR spectra were recorded on a Bruker AM 300 instrument; IR spectra were recorded on a FT-IR Jasco instrument; mass spectra were performed by the Direct Injection System mode with positive Electron Impact using a VG 7070 EQ Instrument. EPR spectra were recorded on a Bruker EMX EPR spectrometer working at the X-band frequency, equipped with a variable temperature BVT 2000 unit (Bruker). HPLC analysis were performed on WATERS 600 E. The detector was a HP 1040 Diode Array Detector. The GC-MSD analysis were performed using a HP 5890 gas chromatograph, interfaced with a quadrupol detector (HP 5970), operating in Electron Impact mode 70 eV.

Oxidations

A solution (40 cm^3) of substrate (0.06 M) and [Co(salen)] (0.006 M) was placed in a glass vessel (100 cm^3) and inserted into an autoclave (250 cm^3). The autoclave was charged with dioxygen (1 MPa) and left at 25 °C for the required time. The solvent was then evaporated under reduced pressure at room temperature and the residue was resolved on a silica gel column with a gradient of ethyl acetate-hexane (3:7 to 7:3) as eluents.

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The quinones **11, 13** (Scheme 2) were identified by comparison with authentical samples (*21*).

The conjugated methylenic aldehyde **12** (Scheme 2), the benzofuran structure **15** and the open ring product **14** (Scheme 3) were identified by the following spectroscopic data, obtained by mass spectrometry, ^{1}H-NMR, ^{13}C-NMR, IR and UV spectroscopies.

Compound **12**: m/z = 178(M^+),149,108,77; ^{1}H-NMR ($CDCl_3$): δ 3.79 (s, 3 H), 5.05 (d, 1 H, J = 2 Hz), 5.25 (d, 1 H, J = 2 Hz), 6.85-7.25 (m, 4 H, aromatics), 9.45 (s, 1 H); ^{13}C-NMR ($CDCl_3$): δ 56.4(CH_3), 110.1(CH_2), 114.8(CH), 118.3(CH), 121.6(CH), 123.7(CH), 143.0(C), 155.0(C), 166.9 (C), 189.5(CH); IR (nujol) = 1703, 1617 cm^{-1}; m.p. = 160 °C.

Compound **14** (isolated after methylation): m/z 472(M^+), 457, 422, 428; ^{1}H-NMR ($CDCl_3$): δ 3.70 (s, 3H), 3.75 (s, 3H), 3.78 (s, 3H), 3.85 (s, 3H), 4.00 (s, 3H), 4.06 (s, 3H), 4.10 (s, 3H), 6.80-7.20 (m, 5H), 6.31 (d, 1H, J=16Hz), 7.75 (d, 1H, J=16Hz); ^{13}C-NMR ($CDCl_3$) δ: 51.8(CH_3), 51.3(CH_3), 52.3(CH_3), 56.8(CH_3), 56.8(CH_3), 56.8(CH_3), 56.8(CH_3), 93.0(CH), 112.8(CH), 112.8(CH), 115.8(CH), 117.1(CH), 119.3(CH), 119.4(CH), 121.4(C), 127.8(CH), 127.9 (C), 141.3(C), 144.6(CH), 146.8(C), 146.9(C), 148.3(C), 158.9.4(C), 166.3(C), 166.4(C); IR (nujol): 1720, 1460, 1149 cm^{-1}; UV (CH_2Cl_2): 228, 300,400 nm.

Compound **15**: m/z = 290(M^+), 359, 277,199; ^{1}H-NMR ($CDCl_3$): δ 3.83 (s, 3 H), 3.83 (s, 3 H), 3.92 (s, 3 H), 6.45 (d, 1H, J=16Hz), 7,75 (d, 1H, J=16Hz), 7.05 (s, 1H), 7.85 (s, 1H), 8.29 (s, 1H); ^{13}C-NMR ($CDCl_3$): δ 52.3(CH_3), 52.3(CH_3), 56.8(CH_3), 115.8(CH), 116.1(C), 118.3(CH), 118.4(CH), 127.2 (C), 130.6(C), 141.3(C), 145.6(CH), 148.3(C), 154.4(CH), 166.3(C), 166.4(C); IR (nujol) 1724, 1644 cm^{-1}; UV (CH_2Cl_2): 230, 265,289 nm.

The conversion (measured as disappearance of starting material) at 30 min, 1 h, 5 h and 48 h was obtained by high performance liquid chromatography-diode array detector (HPLC-DAD) analysis, using calibration curves with biphenyl as internal standard .

The product distribution was determined 48 h from the onset of the reaction by dissolving the residue in a mixture of acetone (20 cm^3) and dimethyl sulphate (0.3 cm^3), and adding K_2CO_3 (0.435 g). After refluxing for 2 h, the solid was filtered out, the solvent evaporated under reduced pressure; the residue was dissolved in CH_2Cl_2 and analyzed by gas liquid chromatography-mass spectrometry (GLC-MS) using biphenyl as internal standard.

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EPR measurements

Deoxygenated solutions were prepared by dissolving [Co(salen)] (0.006 M) in the solvent previously outgassed with a nitrogen stream; the substrate (0.06 M) was then added. Oxygenation with 0.1 MPa dioxygen pressure was performed by bubbling dioxygen for 15 minutes into the deoxygenated solutions. In the reaction conditions (1 MPa dioxygen pressure) aliquots of the solution containing [Co(salen)] (0.006 M) and the substrate (0.06 M) were taken at the following reaction times (min): 5, 10, 15, 20, 30, 45, 60, 120, 180, and immediately cooled in liquid nitrogen in order to slow down the reaction. The EPR spectra were recorded at – 150 °C. The g values were measured by standardization with diphenylpicrylhydrazyl (DPPH) and the relative amounts of the paramagnetic species were obtained by double integration of the resonance line areas.

Results

Oxidations

All the reactions were carried out in chloroform because of the high conversion found in this solvent for the previously reported cobalt-catalyzed oxidation of monomeric compounds (*15*).

Reactions were followed for 48 h. After this time all the substrates had almost completely reacted. The results from previous oxidations of cinnamatea (*15*) are shown in Table I. Tables II and III show the data from the oxidations of the dimeric compounds described in the present paper.

Table I – Conversion % at different reaction times and product distribution % after 48 h for the oxidation in chloroform of the monomeric compounds shown in Scheme 1.

Compound	*Conversion (%)*				*Product distribution (%) after 48 h*	
	30 min	*1 h*	*5 h*	*48 h*	*Benzoic acid derivative*	*Benzaldehyde derivative*
1 (*15*)	60±5	84 ±6	88±4	99±1	**7** 61	**8** 39
2 (*15*)	1±0.5	6±3	37±4	60±4	**9** 17	**10** 43

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Table II – Conversion % at different reaction times and product distribution % after 48 h for the oxidation in chloroform of the β-O-4 type dimeric compounds shown in Scheme 2.

Compound	*Conversion (%)*				*Product distribution (%) after 48 h*	
	30 min	*1 h*	*5 h*	*48 h*	*Quinone derivative*	*Aldehyde derivative **12***
3	90±6	98±5	99±1	99±1	**11** 16	81
4	70±5	83±4	96±3	98±1	**13** 2	20
5	50±10	66±8	70±7	98±1	Not detected	Not detected

Table III – Conversion % at different reaction times and product distribution % after 48 h for the oxidation in chloroform of the β-5 type dimeric compound shown in Scheme 3.

Compound	*Conversion (%)*				*Product distribution (%) after 48 h*	
	30 min	*1 h*	*5 h*	*48 h*	*Fragmentation products*	*Oxygenation product*
6	99±1	99±1	99±1	99±1	**7** 10 **15** 15 **8** 5	**14** 55

All the dimeric compounds showed high conversion within 30 minutes of reaction onset.

The dimeric compounds **3**, **4** showed higher conversion than the monomeric phenols **1**, **2** which have the same substituents *ortho* to the hydroxy group.

Compounds **3**, **4** gave quinones **11**, **13** and the conjugated aldehyde **12** (Scheme 1). Aldehyde **12** has been already isolated as the product of chemical degradation of the β-aryl ether type compound with oxygen in alkaline media and microbiological transformation by Mn Peroxidase (*22*).

Compound **5**, in which a phenolic hydroxyl group lacks, showed the lowest conversion values of the examined dimeric model compounds. Neither quinone nor conjugated aldehyde **12** derivatives were observed; nevertheless, there was the formation of not characterized polymerization products, together with a small amount of vanillin.

From the oxidation of the phenylcoumaran **6**, four main compounds were isolated and identified: **7**, **8**, **14**, **15** (Scheme 3).

EPR investigation

The spectra of [Co(salen)] in a chloroform deaerated solution and under dioxygen (0.1 MPa, 1 MPa) atmosphere were described in a previous paper (*15*).

The reactions of the different substrates with dioxygen (1 MPa) in the presence of [Co(salen)] were monitored by EPR spectroscopy, recording the spectra at – 150 °C on aliquots of the reaction solutions taken at different reaction times and immediately cooled in liquid nitrogen (see experimental). The zero-time solutions were measured under nitrogen atmosphere. Tables IV and V summarize the EPR data of the compounds described in the present paper. For comparison purposes, the spectroscopic behavior of the previously studied E-methyl ferulate **1** and methyl E-4-hydroxycinnamate **2** (*15*) is also reported.

[Co(salen)] – 3-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (3).

The spectrum of the zero-time solution containing [Co(salen)] and the β-O-4 type compound **3**, under nitrogen atmosphere, is the same as that of [Co(salen)]; no interaction with the substrate was observed. The same result was obtained with the other dimeric substrates **4**, **5**, **6**.

After contact with O_2 at 0.1 MPa pressure for 15 minutes, the lines of the phenoxy cobalt radical $[Co^{III}(salen)(RO^-)(RO^\bullet)]$, where ROH is the substrate,

Table IV – EPR data for the oxidation in chloroform of the monomeric compounds reported in Scheme 1.

Compound	*Atmosphere*	*g*	*A (G)*	*Paramagnetic Species*
1 (*15*)	N_2	$g_1 = 3.250$ $g_3 = 1.912$	$A_1 = 93.60$ $A_3 \sim 30$	Planar [Co(salen)]
	O_2 0.1 MPa	As under N_2	As under N_2	Planar [Co(salen)]
		2.0015	18.70	Phenoxy cobalt radical
	O_2 1 MPa	$g_1 = 2.087$	$A_1 = 20.70$	Superoxocobalt derivative
		2.0015	18.70	Phenoxy cobalt radical
2 (*15*)	O_2 1 MPa	2.000	16.20	Phenoxy cobalt radical

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Table V – EPR data for the oxidation in chloroform of the dimeric compounds reported in Schemes 2 and 3.

Compound	*Atmosphere*	*g*	*A (G)*	*Paramagnetic Species*
3	N_2	$g_1 = 3.250$ $g_3 = 1.912$	$A_1 = 93.60$ $A_3 \sim 30$	Planar [Co(salen)]
	O_2 0.1 MPa	As under N_2	As under N_2	Planar [Co(salen)]
		2.002	17.78	Phenoxy cobalt radical
	O_2 1 MPa	2.002	17.78	Phenoxy cobalt radical
4	O_2 1 MPa	Not read	Not read	Superoxocobalt derivative
		2.002	17.58	Phenoxy cobalt radical
5	O_2 1 MPa	Not read	Not read	Superoxocobalt derivative
6	O_2 1 MPa	Not read	Not read	Superoxocobalt derivative
		2.003	18.30	Phenoxy cobalt radical

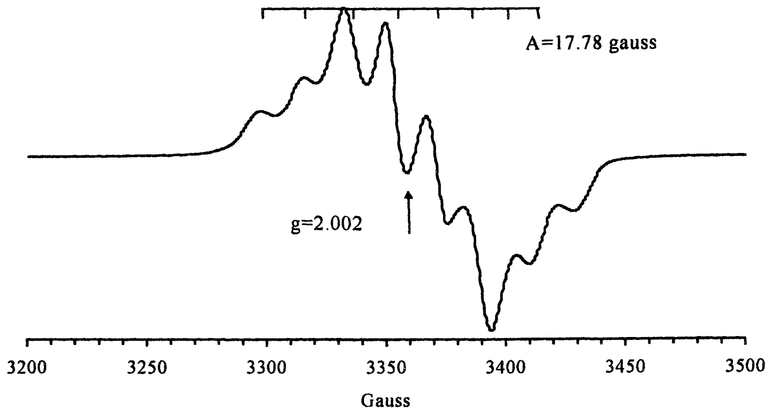

Figure 1 – The EPR spectrum, recorded at – 150 °C, of a chloroform solution of [Co(salen)] (0.006 M) and 3-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol **3** (0.06 M) after contact with O_2 at 1 MPa pressure.

became evident, as were the resonances of [Co(salen)]. Such a spectrum is the same as for the monomeric substrates (*15*) and indicates that [Co(salen)] reacted only in the presence of both dioxygen and the substrate.

At 1 MPa dioxygen pressure, only the phenoxy cobalt radical signal was detected at all reaction times (Fig. 1). It has eight resonance lines centered at g = 2.002, due to the hyperfine interaction with the ^{59}Co (I = 7/2) nucleus, and the low value of the hyperfine coupling constant (A = 17.78 G) suggests that the unpaired electron is mainly located on the substrate.

At reaction onset the amount of radical obtained with compound **3** (Fig. 2, line **3**) was about 3-fold that obtained with E-methyl ferulate **1** (Fig. 2, line **1**), but this amount decreased rapidly and after 30 minutes was almost at the same level as E-methyl ferulate. This suggests that the phenoxy cobalt radical of a β-O-4 type substrate forms more easily, but has a shorter life time than the radical of the monomeric substrate with the same substituents ortho to the hydroxy group.

[Co(salen)] – (E)-methyl-[(2RS-3SR)-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-3-methoxycarbonyl-1-benzofuran-5-yl] propenoate 6.

The β-5 dimeric compound **6** has the same substituents ortho to the hydroxy group as compounds **1** and **3**. After 5 minutes at reaction conditions, a signal attributed to a superoxocobalt derivative was observed, though the components of the magnetic tensors could not be read accurately because of the

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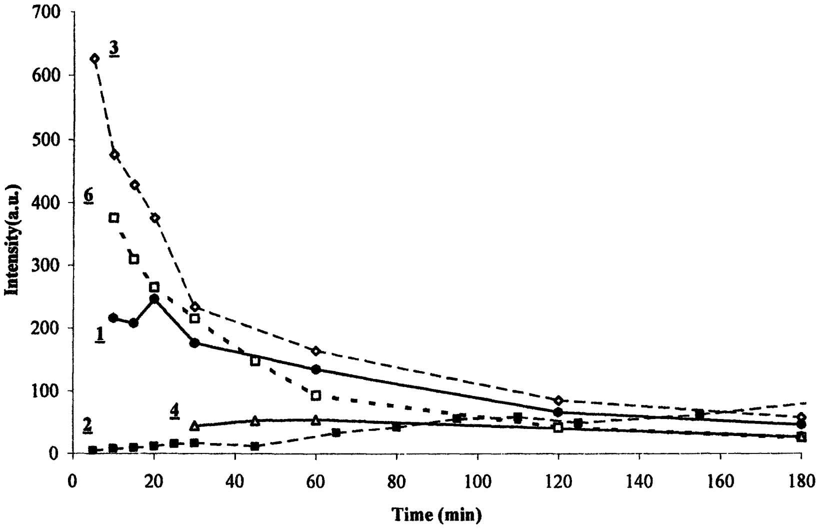

Figure 2 – Trends of intensity (arbitrary units) of resonances *vs.* time (minutes) of the phenoxy cobalt radicals, in chloroform solutions at – 150 °C, of [Co(salen)] (0.006 M) and the following substrates (0.06 M): E-methyl ferulate (**1**), methyl E-4-hydroxycinnamate (**2**); 3 - (4 - hydroxy - 3 - methoxyphenyl) - 2 - (2 - methoxyphenoxy) - 1,3 - propanediol (**3**); 3 - (4 - hydroxyphenyl) - 2 - (2 - methoxyphenoxy) - 1,3 - propanediol (**4**); E-methyl-[(2RS-3SR)-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-3-methoxycarbonyl-1-benzofuran-5-yl] propenoate (**6**).

low intensity and poor resolution of the spectrum. After 10 minutes, the dominant signal was that of the phenoxy cobalt radical (g = 2.003 A = 18.30 G); initially it was about twice as high as the radical obtained with E-methyl ferulate **1**, but it soon decreased rapidly to very small levels (Fig. 2, line **6**). This suggests that also in the case of a β-5 type substrate the formation of the phenoxy cobalt radical is promoted, but the life time of the radical is shorter than that of the radical formed with the corresponding monomeric derivative.

[Co(salen)] – 3-(4-hydroxyphenyl)-2-(2-methoxyphenoxyl)-1,3-propanediol (4).

For this β-O-4 type substrate, which has the same substituents ortho to the hydroxy group as the monomeric substrate **2**, two superimposed signals appeared after 5 minute contact with dioxygen. One has very low intensity and

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is attributed to the superoxocobalt complex, the other is the phenoxy cobalt radical (g = 2.002 A = 17.58 G). After 30 minutes only the signal of the phenoxy cobalt radical was present, both its starting amount and the variation with reaction time being different from the previously examined substrates **3** and **6**: a small constant quantity was detected for each reaction time (Fig. 2, line **4**). Nevertheless, also in this case, the phenoxy cobalt radical of the dimeric substrate formed in higher amount and was less stable than that of the corresponding monomeric substrate (Fig. 2, line **2**).

[Co(salen)] – 3-(4-methoxyphenyl)-2-(2-methoxyphenoxyl)-1,3-propanediol (5).

For this β-O-4 type substrate, where the aromatic hydroxyl is replaced by a methoxy group, only the signal attributable to the superoxocobalt complex was present at each reaction time. This signal has low intensity and is poorly resolved.

Discussion

Previous papers concerning the oxidation of monomeric phenols by molecular oxygen (*14*, *15*) had suggested the following mechanism on the basis of EPR results:

$[Co^{II}(salen)] + ROH + O_2 \rightarrow [Co^{III}(salen)(ROH)(O_2^{-})]$ (A)
$[Co^{III}(salen)(ROH)(O_2^{-})] + ROH \rightarrow [Co^{III}(salen)(ROH)(RO^{\bullet})] + HO_2^{-}$ (B)
$[Co^{III}(salen)(ROH)(RO^{\bullet})] + HO_2^{-} \rightarrow [Co^{III}(salen)(RO^{-})(RO^{\bullet})] + H_2O_2$ (C)

First, the aromatic hydroxyl of ROH and dioxygen co-ordinate with [Co(salen)] with the formation of the EPR active superoxocobalt derivative, $[Co^{III}(salen)(ROH)(O_2^{-})]$; this first step is favored by the electron-donor properties of the phenol (*23*).

Then the resulting superoxocobalt derivative reacts with another phenol ligand, the O_2^{-} abstracting a hydrogen atom from ROH; for this step the more basic the co-ordinated phenol, the greater the possibility that the superoxide abstracts a hydrogen atom from ROH (*24*). An EPR active phenoxy cobalt radical, $[Co^{III}(salen)(RO^{-})(RO^{\bullet})]$, forms and its stability depends on the ability of the phenol to delocalize the unpaired electron.

At the end the $RO^{\bullet}$ radical probably undergoes decomplexation from cobalt and is further attacked by O_2 giving the final reaction products.

The same type of paramagnetic species observed during the oxidation of monomeric cinnammates **1** and **2** were detected with the dimeric compounds **3**,

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4, **5** and **6**: the superoxocobalt derivative was observed in the first reaction minutes of compounds **4** and **6** and was the only paramagnetic species at each reaction time of compound **5.** Compound **3** showed only phenoxy cobalt radical at each time of reaction.

For the formation of the phenoxy cobalt radical, it is necessary that the aromatic hydroxyl co-ordinates with [Co(salen)]; in fact the radical does not form in the case of compound **5**.

Step B (the formation of the phenoxy radical) depends on the electron donor character of the ROH ligand (*24*). In fact in the first minutes of reaction the amount of the phenoxy cobalt radical increased with the electron donor character of the ligands (**4** << **3** ~ **6**), a trend expected from the substituents on the phenolic ring. The same trend is present in the substrate conversion after 30 minutes from the onset of reaction.

The dimeric compounds react faster than the corresponding resonance stabilized monomeric esters **1** and **2** because of their stronger nucleophilic character, in particular phenylcoumaran **6** is completely degraded after 30 minutes. Non phenolic model compounds are not degraded, but nevertheless they react to give principally polymerization products.

The oxidative degradation of phenolic dimeric model compounds by molecular oxygen catalyzed by [Co(salen)] has given good results, the reaction is fast and interesting low molecular weight compounds such as quinones **11, 13,** vanillin **8** and α-(2-metoxyphenoxy)-acrolein **12** were isolated with good yields.

This oxidative system can be developed to degrade real waste lignin because the work up is easy, the amount of cobalt used is low and the cobalt can be recovered at the end of reaction.

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Chapter 12

A Biomimetic Approach to Lignin Degradation

Metalloporphyrins Catalyzed Oxidation of Lignin and Lignin Model Compounds

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An overview of the state of the art for the use of synthetic metalloporphyrins in the catalytic oxidation of lignin and lignin model compounds is presented. The biomimetic oxidation of 5-5' condensed and diphenylmethane lignin model compounds with several water soluble anionic and cationic iron and manganese porphyrins in the presence of hydrogen peroxide is described. Manganese porphyrins were found more effective in degrading lignin substructures than iron porphyrins. Among them the cationic manganese *meso*-tetrakis(N-methyl-pyridinio) porphyrin pentaacetate [TPyMePMn($CH_3COO)_5$], never used before in lignin oxidation, proved to be the best catalyst. The catalytic activity of porphyrins in hydrogen peroxide oxidation of residual kraft lignin was also investigated. TPyMePMn($CH_3COO)_5$ was able to perform the most extensive degradation of the lignin structure, as demonstrated by the decrease of aliphatic hydroxyl groups and increase of carboxylic acids, as measured by quantitative ^{31}P-NMR. No significant condensation reactions occurred during manganese porphyrin catalyzed oxidations of residual kraft lignin, while in the presence of iron porphyrins an increase of condensed moieties was detected.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Introduction

A main goal in the development of catalytic processes in chemistry is the mimicking of biological transformations. The objective is to perform reactions in a context isolated from the biological one, and to use a catalyst with a minor cost than the enzyme itself. A modern approach to the development of new catalytic systems goes beyond these former intentions. The development of synthetic catalysts which can mimic the course of biological processes is proposed. Such systems should also provide a wider margin of catalyst stability, conversion yields, selectivity in the reaction, and lower substrate selectivity than the enzyme itself. The goal of such a process is the development of a system that is both environmentally friendly and economically suitable for the scale up to plant dimensions.

In the paper production processes, environmental concerns have prompted the study of pulping and bleaching sequences avoiding the use of chlorinated compounds. Several totally chlorine free (TCF) processes have been developed, for example by the use of oxygen, hydrogen peroxide, and ozone respectively.(1) However their major drawback consists in a lack of selectivity in the degradation of lignin, which leads to the partial degradation of the cellulose contained in pulps, and ultimately in a lower final yield. The reason for this lack of selectivity in the oxidation reactions, is due fundamentally to the formation of common radical intermediates such as hydroxyl radicals, that are able to attack both cellulose and lignin.(2)

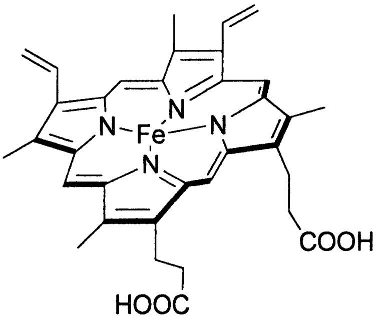

Figure 1. Fe(III) protoporpyrin IX

The selective removal of lignin in wood is accomplished, in nature, by the white-rot basidiomycetes fungi. Such fungi are able to produce three classes of ligninolitic enzymes: laccases, lignin peroxidases (LiP) and manganese

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dependent peroxidases (MnP).(3,4,5) The former are oxygenases with an active site containing four copper atoms. The oxidation of phenolic lignin subunits is performed, in the presence of oxygen, by the generation of phenoxy radicals. LiP and MnP are enzymes that can perform the heterolytic oxygen transfer from hydrogen peroxide to the active center. MnP is able to oxidize only phenolic lignin substructures, while LiP, due to its higher redox potential, can oxidize also non-phenolic substrates. The active site of these enzymes is constituted by a heme center, the protoporphyrin IX (iron-PPIX)(Figure 1). (6)

Synthetic metalloporphyrins are biomimetic catalysts that can yield highly oxidized metallo-oxo species. They have been used as lignin peroxidase models, and their potentiality for lignin degradation has been a subject of several studies. (7-21)

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Discussion

In the presence of hydrogen peroxide, the active center of LiP and MnP performs a one-electron oxidation of the lignin aromatic moieties.(6,22) The catalytic cycle consists in a two electron oxidation of Fe (III) protoporphyrin IX (high spin) to give a highly reactive oxo-iron (IV) protoporphyrin IX π-cation radical, the LiP I complex. The LiP compound I is then reduced to the initial state by two different one electron reductions by the substrates (Figure 2). LiP is a fragile enzyme. When exposed to an excess of hydrogen peroxide (more than 20 eq.), it is subject to inactivation by overoxidation, and gives the inactive form LiP III (Fig. 2).The protein scaffold around the active center provides stabilization of the metal complex, protection of the active site from possible overoxidation processes, and water solubility.

When synthetic metalloporphyrins are used as biomimetic catalysts in the presence of hydrogen peroxide, several side reactions can occur. The peroxidic bond can undergo homolytic scission to yield Fe (IV)-OH and hydroxyl radical in a Fenton like fashion. This reactivity is more significant in the presence of iron complexes and hydrogen peroxide as oxygen donor. A second molecule of peroxide may react with the metal oxo complex in a catalase like fashion to yield the formation of H_2O and O_2, and ultimately the degradation of the active oxidant species. The metal oxo complex may react to yield μ-oxo dimers. (7)

In Nature the polypeptidic envelope of the enzyme protects the active site from side reactions, and activates it. In fact, the heterolytic cleavage of the peroxidic bond is subject to acid catalysis. The proxymal His residue activates the complex to heterolytic cleavage by enhancing the electrophilic character of the oxygen atom, and reducing the strength of the metal-oxygen bond. the addition of a nitrogen base such as pyridine or imidazole to the oxidizing solution can mimic this situation. (7) The manganese porphyrins are not strictly biomimetic systems of LiP, since the natural enzyme active site is an iron complex. However the use of manganese complexes could override many reactivity problems. More specifically, manganese porphyrins are usually more reactive than the iron porphyrins;(7) they form single adducts with nitrogen bases and in this way can be easily activated. Manganese shows a minor tendency than iron to undergo the homolytic cleavage of the peroxidic bond.

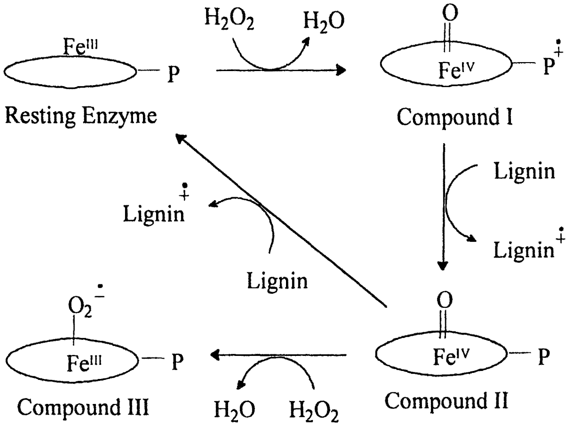

Figure 2. Catalytic cycle of lignin peroxidase

Oxidation of lignin model compounds

Early studies on metalloporphyrins as ligninase biomimetic catalysts were based on the use of iron-PPIX. (8-11) Shimada et al. (11) reported that iron PP IX catalyzes the oxidation of β-1, β-O-4 and β-5 lignin model compounds thus mimicking ligninase. Also, the regiospecifity found in iron-PPIX oxidation of veratryl alcohol was the same observed in LiP; this showed that the regiospecificity of oxygenation is not necessarily governed by the protein moiety of ligninase.(Figure 3)

However, simple metallo porphyrins suffer from the major disadvantage of being unstable in the presence of excess oxidants. Their lability is due either to self-destruction or to the formation of inactive μ-oxo complexes. (7,12) Sterically protected porphyrins were then studied. The presence of phenyl groups in the *meso* positions of the porphyrin ring highly increased its stability to

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oxidants. A series of differently ring-substituted catalysts with increased redox potential and /or water solubility were then analyzed.(7,12) The presence of sulphonic groups highly increased water solubility, while the introduction of halogen atoms onto the phenyl ring or the β-positions enhanced the resistance to oxidants.

Several studies have been reported aimed at evaluating the possible use of such catalysts as ligninase models. Veratryl alcohol is a typical non-phenolic lignin model and a secondary metabolite that acts as a mediator in the ligninase oxidation of lignin.

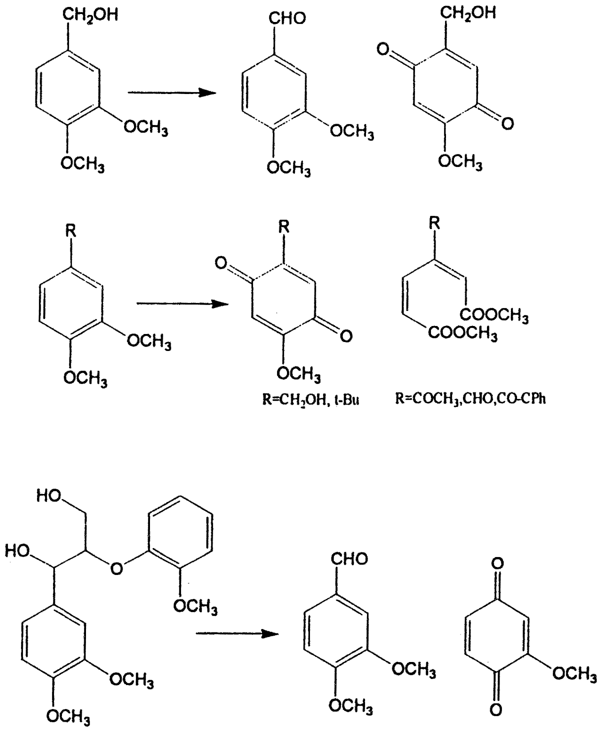

Figure 3. Oxidation of lignin model compounds catalyzed by metallo porphyrins and some of the oxidation products (nonexhaustive list).

When submitted to oxidation with iron *meso*-tetraphenylporphyrin (FeTPPS) or manganese *meso*-tetraphenylporphyrin (MnTPPS) (H_2O_2 or $KHSO_5$) it was oxidized to veratraldehyde and *p*-quinone (Figure 3). (13) The presence of different iron or manganese porphyrin did not affect the nature of the oxidation products. As oxygen donors were employed H_2O_2, MMP, *t*-BuOOH, *m*CPBA, $KHSO_5$. (13-16) Hydrogen peroxide would be the oxidant of choice for

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its low cost and low environmental impact. However H_2O_2 show the tendency to undergo homolytic cleavage of the peroxidic bond more than other oxygen donors. The oxidation of methoxy arenes carrying electron-withdrawing groups such as -CHO, -$COCH_3$ follows a dramatically different reaction pathway. (14) In fact in this case the different distribution of the electron density, due to the captodative effect of the substituents, yields products of aromatic ring cleavage, the muconate derivatives (Figure 3).

Dimeric lignin model compounds have also been studied. The β-O-4 linkage is by far the most common substructure in lignin. (13-17) The oxidation of 1-(4-methoxy-3-methoxyphenyl)-2(2-methoxyphenoxy) propan-1, 3-diol a β-O-4 dimeric lignin model, when performed in iron *meso*-tetrakis(2,6-dichloro-3-sulphonatophenyl) porphyrin chloride (TDCSPPFeCl), *t*BuOOH in H_2O/CH_3CN, yielded products of side-chain oxidation and cleavage. In the presence of TPPS and $KHSO_5$ or H_2O_2, the *p*-quinone was also obtained (Figure 3). Mansuy and Dolphin studied the reactivity of diarylpropane units. (14,17) In the presence of TDCSPP, TPPS, *meso*-tetrakis(2,6-dichlorophenyl) porphyrin (TDCPP), and *meso*-tetrakis(pentafluoro-β-tetrasulfonatophenyl) porphyrin (TF_5PS_4P), the main products obtained were side chain oxidation products, and the corresponding *p*-quinone.

The phenyl coumaran (β-5) substructure constitutes about 9-12% of the substructure in softwood lignin, while the 5-5' units are about 9.5-11%. The study of the degradation of such lignin subunits showed that both of them are actively oxidized by metalloporphyrins in the presence of *t*-BuOOH or H_2O_2. The products obtained are again side-chain oxidation products, muconate derivatives from aromatic ring cleavage reactions, and *p*-quinones. (17)

A major drawback to the use of iron porphyrins in lignin oxidation is that their maximum activity is at pH about 3. At acid pH the homolytic cleavage of the peroxidic bond is favored. The reaction pattern observed could be due both to the presence of the high valence Me (IV) $P^{+\bullet}$ and to the formation of hydroxyl radicals. Mn porphyrins show their maximum activity at pH 6. (7) Furthermore, manganese is less prone than iron to catalyze the Fenton reaction.

We designed a protocol to evidenciate the possible reactivity of several iron and manganese porphyrins toward condensed lignin subunits. (18) Residual lignin after kraft pulping is in fact heavily modified with respect to native lignin. Residual Kraft lignin contains significant amounts of 5-5' biphenyl and diphenylmethane substructures. (19) The oxidative behavior of porphyrins on isolated lignins and residual kraft lignins has not been much explored. (9,11,20,21)

The oxidation pathway of 5-5' biphenyl and diphenylmethane lignin models, in the presence of cationic and anionic water-soluble porphyrins, using hydrogen peroxide as oxidant has been recently reported. (18) The oxidative efficiency of anionic manganese and iron TDCSPP, and TSPP was compared on the basis of

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the oxidation extent of the tested models. The catalytic activity of the cationic manganese *meso*-tetrakis(N-methyl-pyridinio) porphyrin pentaacetate [TPyMePMn($CH_3COO)_5$] on lignin model compounds was determined at two different pH values: 3 and 6.

*Oxidation of 2,2',3,3'-tetramethoxy-5,5'-dimethyl biphenyl **1** and 2,2', 3,3'-tetramethoxy-5, 5'-dimethyl diphenylmethane **5**.*

5-5' Condensed model **1** was oxidized in the presence of hydrogen peroxide and several Mn or Fe porphyrins at pH 3 or 6. More specifically, TDCSPPMnCl and TSPPMnCl were used at pH 6, while, in accord to the maximum activity pH previously reported (16), TDCSPPFeCl was used at pH 3. TPyMePMn ($CH_3COO)_5$ was used at pH 3 and 6 in order to test the pH of its optimal catalytic activity. The same reactivity pattern was observed under all the tested experimental conditions, the main oxidation products detected being in every case the *p*-quinones **2** and **3** (Scheme 1, Table I).

Table I. Conversion of lignin model compound 1 and product yields.

	MnTPyMeP pH3^a	*MnTPyMeP pH6*^{b}	*MnTSPP*^{c}	*Mn TDCSPP*^{d}	*Fe TDCSPP*^{e}
Residual **1** (%)	27.1	41.2	72.7	49.9	55.3
2 (%)	51.2	53.9	21.6	43.9	Trace
3 (%)	2.04	2.22	3.14	2.55	3.14
4 (%)	1.14	1.19	1.57	1.90	2.17

a: oxidation in the presence of TPyMePMnAc$_5$ at pH3; b: oxidation in the presence of TPyMePMnAc$_5$ at pH6; c: oxidation in the presence of TSPPMnCl at pH6; d: oxidation in the presence of TDCSPPMnCl at pH6; e: oxidation in the presence of TDCSPPFeCl at pH3. Reprinted from *Bioorg. Med. Chem.* **1999**, *7*, 1897, with permission from Elsevier Science

In analogy with the general oxidative mechanisms previously reported for porphyrin-catalyzed reactions, the following pathway could be hypothesized. The formation of compound **2** can be rationalized on the basis of the electronic effect exerted by the methyl group. The intermediate radical cation **1**$^{+\bullet}$, that is generated during the first one electron oxidation step, could be further oxidized by the highly electrophilic species (P) Me^{IV}=O. The site of oxidation is determined by the distribution of the electronic density on the aromatic ring,

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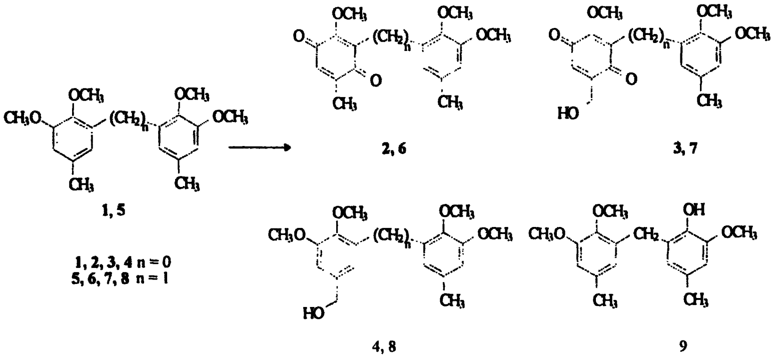
which is in turn modulated by the electronic effect of the substituent at the C-5 position (15). The electron donating effect of the methyl group could direct the second oxidation step at the C-6, and yield the corresponding *p*-quinone **2** (Scheme 2). This behavior had been previously reported on monomeric compounds carrying electron-releasing substituents. A side-chain oxidation reaction yielded the product of benzylic oxidation **4**. The occurrence of side chain oxidation reactions had been previously described (14,16,17). The *p*-quinone **3** could be formed, in analogy with **2**, by the further oxidation of **4**.



Scheme 1. Products obtained from the oxidation of lignin model compounds 1 and 5. Reprinted from Bioorg. Med. Chem. ***1999****, 7, 1897, with permission from Elsevier Science*

In Table I are reported the yields of products **2**, **3**, **4** and the amount of **1** recovered after reaction under the different experimental conditions examined. A comparison between TDCSPPMnCl and TDCSPPFeCl showed that the manganese porphyrin was able to perform a more extensive oxidation of model compounds **1** and **5** than the iron porphyrin. This could be due to a higher stability under oxidative conditions rather than to an intrinsic higher reactivity toward the lignin moieties. (16) Under such experimental conditions the homolytic cleavage of the peroxidic bond in a Fenton like fashion is in competition with the heterolytic reaction (12,23,24).

The anionic porphyrin TSPPMnCl proved to be the less active catalyst. In fact it lacks of any electron withdrawing substituent, which can enhance the redox potential. The corresponding TDCSPPMnCl, in which two chlorine atoms have been added to each phenyl ring, proved to be more reactive.

TPyMePMn $(CH_3COO)_5$ showed a higher conversion rate at pH 3 than 6. (Table I). However the amount of products **2**, **3**, and **4** were comparable. Probably, when the reaction was performed at pH 3, the higher acidity of the medium underwent further radical coupling reactions yielding higher molecular

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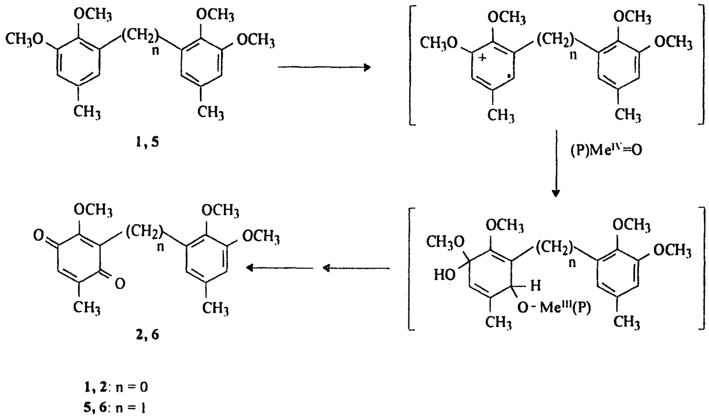
weight condensed products (25). Under both the experimental conditions TPyMePMn $(CH_3COO)_5$ was found to be more effective in the degradation of **1** than the anionic catalysts.

The oxidation of the diphenylmethane model compound **5** showed an analogous reaction pathway with *p*-quinone **6** being the main product recovered (about 10 % yields) (Schemes 1, 2).



Scheme 2. Proposed reaction pathway for the formation of p*-quinones* ***2*** *and* ***6****. Reprinted from* Bioorg. Med. Chem. ***1999****, 7, 1897, with permission from Elsevier Science*

Bleaching of kraft pulps.

Paszczynski (9) studied the treatment of kraft pulps in the presence of *t*BuOOH with a variety of natural and synthetic porphyrins. The biomimetic system was able to effectively bleach the pulps. After treatments the pulps were found essentially lignin free, although a little loss occurred in the cellulose content. Extensive delignification was obtained using the water-soluble catalysts FeTDCSPP, iron meso-tetrakis(2,6-dichloro-3-sulfonatophenyl)-β-octachloroporphyrin ($FeTDCSPCl_8P$), and iron meso-tetrakis(4-sulfonatophenyl)-β-octachloroporphyrin $FeTSPCl_8P$ (21). The use of the corresponding manganese porphyrins did not significantly alter the lignin degradation extent (K number). However, a decrease in the viscosity of pulps, due to cellulose degradation, was evident in all the experiments. Possible technological applications require a better control of the oxidative reactions and milder reaction conditions. From this viewpoint the elucidation of the oxidation mechanism on lignin is of pivotal importance.

Oxidation of lignin

Kurek (20) reported a study on the structural modification induced on milled wood lignin and extractive free wood by $FeTF_5PS_4P$ in the presence of hydrogen peroxide. It was established that demethylation, aromatic ring cleavage and side chain oxidation reactions occurred.

In order to elucidate the reactivity pattern of iron and manganese porphyrins on kraft pulps we designed a study on the oxidation of softwood residual kraft lignin with hydrogen peroxide and different metalloporphyrins. More specifically, the different activity of Mn and Fe porphyrins was tested on FeTDCSPP and MnTDCSPP. The effect of the chlorine atoms was studied by the comparison of MnTSPP and MnTDCSPP. Moreover, the catalytic effect of MnTMePyP, a water-soluble cationic porphyrin, never used to date in the oxidation of lignin or lignin models was studied.

The oxidation of residual kraft lignin with TDCSPPMnCl and TSPPMnCl was carried out at pH 6, while the oxidation with TDCSPPFeCl was performed at pH 3. Such values were chosen according to the maximum activity of iron and manganese porphyrins previously found at different pH values (16). The oxidations with TPyMePMn $(CH_3COO)_5$ were performed both at pH 3 and 6.

The structural modifications induced on the polymer were quantitatively determined by ^{31}P-NMR spectroscopy (26-31). In Table II the quantitative data obtained from the spectra of lignin before and after the different treatments are reported. The comparison of the efficiency of TDCSPPFeCl and TDCSPPMnCl in oxidations performed at pH 3 and 6 showed, under both the experimental conditions, a decrease of aliphatic OH groups. This shows the occurrence of side-chain oxidation reactions. The increase of COOH units further confirms this behavior. In the case of TDCSPPMnCl such increase was found too high to be explained only on the basis of side chain oxidation reactions. Thus the presence of aromatic ring cleavage reactions cannot be ruled out.

The effect of H_2O_2 porphyrin mediated oxidation on lignins, was also evident on the modification of the phenolic groups. In the presence of TDCSPPMnCl only a decrease of guaiacyl groups was observed, while in the presence of TDCSPPFeCl an increase in the NMR chemical shift region of condensed structures was evident. Such structures have been previously assigned to β-5, 4-O-5 and 5-5' condensed units (32). This seems to indicate a different reaction pathway for iron and manganese porphyrins, with the latter being able to carry out an oxidative process with a low amount of coupling reactions. The

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coupling reactions that occur during the oxidations catalyzed by iron porphyrins could be due to the formation of hydroxyl radicals generated by the homolytic cleavage of hydrogen peroxide. Furthermore, the overall oxidation was found more extended on lignins treated with TDCSPPMnCl than with TDCSPPFeCl. This behavior is in accord with the previously examined extent of oxidation of lignin model **1**.

A comparison of the reaction course in the presence of the cationic porphyrin TPyMePMn $(CH_3COO)_5$ at different pH values showed that the oxidation of lignin was improved at pH 6 rather than pH 3 (Table II). In fact the treatments performed at pH 6 resulted in a net increase of the carboxylic units and decrease of aliphatic hydroxyl groups. Since the COOH increase was found higher than the aliphatic OH decrease, the carboxylic units cannot be formed only by side-chain oxidation reactions, but also aromatic ring cleavage processes could occur.

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Table II. Distribution of aliphatic, phenolic and carboxylic hydroxyl groups in residual Kraft lignins before and after porphyrin catalyzed oxidations.

lignin/treatment	*Aliphatic OH*	*Condensed phenolic OH*	*Guaiacyl OH*	*COOH*
		(mmol/g)		
$TPyMePMnAc_5$ pH 3	1.78	0.97	1.04	0.35
$TPyMePMnAc_5$ pH 6	1.64	0.91	1.09	0.65
TSPPMnCl pH 6	1.79	0.93	1.07	0.30
TDCSPPMnCl pH 6	1.76	0.90	0.98	0.48
TDCSPPFeCl pH 3	1.75	1.01	1.14	0.41
Kraft lignin[a]	1.94	0.92	1.27	0.27
H_2O_2 pH 3[b]	1.78	0.94	1.02	0.36
H_2O_2 pH 6[c]	1.80	0.89	1.08	0.34

Data obtained from quantitative ^{31}P-NMR spectra of samples phosphytylated with 2-chloro-4, 4,5,5-tetramethyl-1, and 3,2-dioxaphospholane. a: starting reference sample; b: control experiment in the absence of porphyrins at pH 3; c: control experiment in the absence of porphyrins at pH 6. Reprinted from *Bioorg. Med. Chem.* **1999**, *7*, 1897, with permission from Elsevier Science

The comparison of anionic and cationic water-soluble Mn porphyrins TSPPMnCl and TPyMePMn $(CH_3COO)_5$ respectively, showed an increased efficiency in the cationic TPyMePMn $(CH_3COO)_5$ toward the oxidation of lignin (Table II). The efficiency was evaluated on the basis of the aliphatic OH decrease and COOH increase. To our knowledge this is the first example of the use of a cationic porphyrin for lignin oxidation. It is worth noting that in all the oxidations carried out at pH 6 the amount of condensed phenolic substructures did not vary significantly. When the oxidations were performed at pH 3, processes occurring with condensation reactions were found active. In particular the iron porphyrin TDCSPPFeCl was more prone to undergo condensation than TPyMePMn $(CH_3COO)_5$.

Concluding remarks

Anionic and cationic water-soluble manganese porphyrins are more effective in degrading residual kraft lignin and lignin substructures than iron porphyrins. This effect could be due to a higher stability of Mn porphyrins under oxidizing conditions. Among these the cationic TPyMePMn $(CH_3COO)_5$, never used before in lignin oxidation, showed to be the best catalyst.

Both Fe and Mn porphyrins are able to oxidize such recalcitrant condensed lignin substructures as those represented in models **1** and **5**, and yield *p*-quinones that in turn can be easily cleaved. However during hydrogen peroxide oxidation of residual kraft lignin in the presence of TDCSPPFeCl further condensation reactions are likely to occur. The final products are modified lignins with increased amounts of condensed substructures. On the other hand, residual kraft lignin treated with Mn porphyrins at pH 6 did not show any significative increase in the condensed units. These findings suggest that the overall lignin oxidation by H_2O_2 in the presence of Fe and Mn porphyrins follows different reaction pathways. Formation of hydroxyl radicals by the homolytic cleavage of the peroxidic bond of H_2O_2 can occur, especially in the case of iron porphyrins. This reaction pattern is in competition with the heterolytic reaction. The suppression of condensation reactions that occurs during the oxidations of residual kraft lignin catalyzed by manganese porphyrins prompts at a further evaluation of their applicability in lignin degradation processes.

Points to ponder and future directions

Despite the progress achieved in the synthesis of metallo porphyrins resistant to oxidation, a major drawback is still present to their applicability. Metallo porphyrins are, to date, expensive catalysts. Their potential use in lignin oxidation is bound to the possibility of a further increase of their stability toward hydrogen peroxide. Furthermore, the need to recover and recycle the catalysts after their use is also pressing. A possible approach to the development of such new catalysts has been attempted taking into consideration that these two aims could be reached by immobilization of the catalyst onto a suitable support. Several approaches of this kind are possible ranging from organic synthetic

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polymers to biopolymers or to inorganic matrices. To date several procedures for metallo porphyrins immobilization onto inert matrices have been developed. (8,13) However the reactivity of the immobilized systems is usually lower than the soluble porphyrin. Meunier reported the oxidation of veratryl alcohol and a β-O-4 model dimer with FeTPPS and MnTPPS immobilized on the exchange resin Amberlite IRA 900 EGA using H_2O_2 or $KHSO_5$ as oxygen donor. The system immobilized-MnTPPS $KHSO_5$ showed to be the most active. (14)

Much work is still to be done on the degradation of lignin by immobilized metalloporphyrins. From this view-point the use of hydrophobic membranes, that allow the equimolar transfer of H_2O_2 and substrate to the catalyst, and avoid porphyrin bleaching is a promising technique. Smectite clays provide negatively charged layers large enough for the accommodation one porphyrin and one substrate molecule only. Thus, immobilization onto clays would prevent from the formation of μ-oxo dimers. The possibility to use clays as support is also a promising approach.

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Chapter 13

One-Electron Oxidation Activity of Dendritic Porphyrin in Nonaqueous Media

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An artificial peroxidative catalyst for the oxidation of aromatic compounds in nonaqueous media was designed and prepared. A dendritic porphyrin containing *meso*-tetra(4-carboxyphenyl) porphine (TCPP)-iron complex was synthesized using 3,5-dihydroxybenzyl alcohol as a building block with each dendron extended to the fourth generation (G4 Fe-TCPP). This dendrimer was chosen in order to introduce an interface between TCPP and a solvent. This complex was capable of effectively oxidizing either phenolic or nonphenolic aromatic compounds in hydrophobic solvents. Kinetic studies revealed that the catalysis followed an enzyme-like reaction mechanism. The structure/function relationship was studied utilizing kinetic and spectroscopic techniques and a method based on computational chemistry. It was thus shown that the catalytic performance of G4 Fe-TCPP was due to the ester linkages between dendrons and TCPP, which lowered the barrier energy for the free rotation of the dendrons.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Lignin is the most abundant renewable aromatic polymer, and is known as one of the most recalcitrant biomaterials on earth (1, 2). The biodegradation of lignin plays a key role in the carbon cycle of the biosphere (3-5). Only white-rot basidiomycetes have been known to be responsible for the complete mineralization of this polymer. Besides studies on the ligninolytic system, many application studies have been focused on the utilization of white-rot fungi. *Phanerochaete chrysosporium* and *Coriolus versicolor* have been shown to degrade environmentally persistent aromatic pollutants (6-11). Among the catalysts involved in fungal degradation of pollutants, lignin peroxidase (LiP) has been shown to play an important role toward initiating the degradation of aromatic compounds.

LiP is a unique peroxidase, catalyzing the direct one-electron oxidation of aromatic rings, which allows this enzyme to attack nonphenolic aromatic pollutants such as polychlorinated dibenzo-*p*-dioxin, polychlorinated biphenyls, and polycyclic hydrocarbons. However, it has been hypothesized that the reduction potential of highly chlorinated aromatic rings should be higher than that of LiP, because chloro substituents are electron-withdrawing groups. Furthermore, the more chlorinated the compounds are, the more hydrophobic the aromatic compounds become. In this study, we have designed an artificial enzyme which has a higher reduction potential than that of LiP and functions in hydrophobic organic solvents. One of us has already reported that LiP catalyzes the oxidative degradation of dichlorinated dibenzo-*p*-dioxin with a release of chloride ion (8, 12). Consequently, we prepared a LiP-mimicking catalyst functioning in organic solvents.

STRATEGY

LiP type catalyst: To mimic a LiP type one-electron oxidation, porphyrin derivatives were chosen as the catalytic core.

Reduction potential higher than LiP: A series of porphyrins were examined to oxidize veratryl, anisyl, and benzyl alcohols. *meso*-Tetra (4-carboxyphenyl) porphine-iron (Fe-TCPP) complex was found to be the most effective one-electron oxidizer among those examined. However, Fe-TCPP was not soluble in hydrophobic solvents. Furthermore, it was easily inactivated by peroxide during the reaction. It was thus decided to chemically modify Fe-TCPP. Four carboxylic groups of TCPP were advantageous for further synthetic reactions.

Dendrimer: Kinetic analyses showed that Fe-TCPP catalyzed the oxidation of either phenolic or nonphenolic compounds as a chemical catalyst but not as an enzymatic catalyst. For more effective reactions, an enzymatic catalyst is preferred. To turn Fe-TCPP into an enzymatic catalyst, the interface between the active core and the solvent was designed, which could result in the introduction of pre-equilibrium resembling the formation of enzyme-substrate complex. The dendrimer is a globular-shaped, nanoscopic sized, hyper-branched macromolecule. The synthesis of a porphyrin covalently encapsulated

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into a huge dendritic cage has been reported (13). The dendrimer was thought to be a superior architecture, introducing an interface between the porphyrin and the solvent. Since the interior nanoenvironment is different from the exterior solvent environment, the molecular selectivity for substrate incorporation into the dendrimer can be expected to be augmented, thus providing for substrate specificity.

EXPERIMENTAL

Dendrons were synthesized convergently using methyl 4-hydroxymethylbenzoate as a chain end block and 3,5-dihydroxybenzyl alcohol as a building block. The general procedure is a bromination of an alcoholic hydroxyl group followed by an alkylation with 3,5-dihydroxybenzyl alcohol, as previously described (14). Four dendrons were esterified to TCPP (four carboxylic groups) using a water-soluble carbodiimide. TCPP with a mono-aryl layer was designated generation 1 (G1). Consequently, G2, G3, and G4 TCPPs were also prepared (Fig. 1). These products were purified using flash chromatography. An Fe ion was incorporated after dendron-TCPP complexes were prepared.

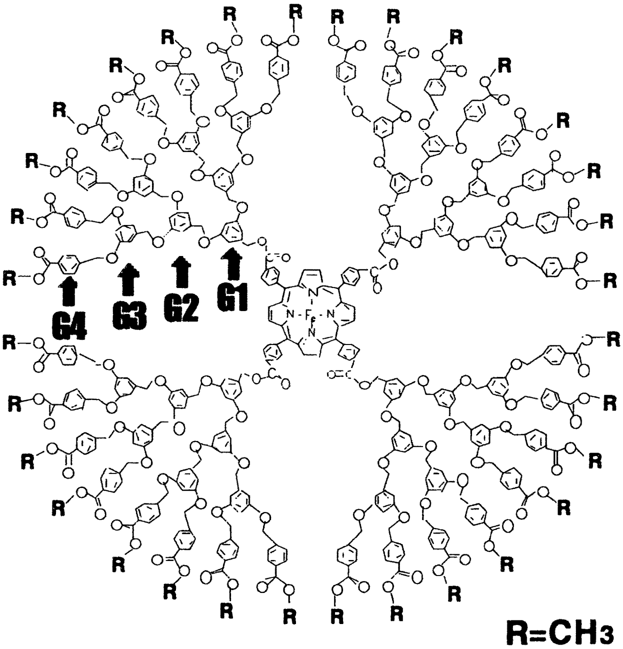

Fig. 1 Chemical Structure of Dendritic Porphyrin

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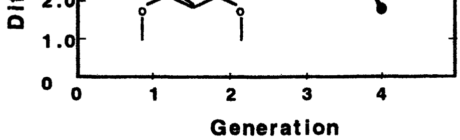
The optimized structure of Gn Fe-TCPP (n = 1-4) was obtained in terms of computational chemistry using molecular mechanics and molecular dynamics. These optimizations were calculated using an MM3 force field.

Electron absorption spectra were recorded on a Perkin-Elmer λ-19 spectrophotometer. ^{1}H-NMR spectra were recorded on JEOL JNM-AL400 (400 MHz). GCMS, FAB-MS, and TOF-MS were taken using JEOL Automass 15, JEOL JMS-LX1000, and PE Biosystems Voyager, respectively. The water content of the organic solvents was determined using the Karl Fischer potentiometric titration using a Mitsubishi moisturemeter CA-05.

RESULTS AND DISCUSSION

The identification of the products was confirmed using GCMS, TOF-MS, and ^{1}H-NMR. Fe incorporation was confirmed by FAB-MS (data not shown).

Interior Environment of Dendritic Porphyrins: The absorption spectra in chloroform showed that the Soret bands of Gn Fe-TCPP (n = 1-4) were red shifted from the Soret peak of free Fe-TCPP. The shift increased as the number of generation increased (Fig. 2). The Soret band of G4 Fe-TCPP in chloroform was very close to that of Fe-TCPP in 1,3-dimethoxybenzene, a monomeric model for a building block of each dendron. These observations strongly suggested that G4 dendrons encapsulated Fe-TCPP, providing a new interface between TCPP and the solvent. Furthermore, the microenvironment of the dendrimer interior is different from the exterior environment of the solvent.



Difference = λ max (Fe-TCPP in 1,3-DMB) - λ max (in $CHCl_3$)
Inset: the chemical structure of the building block

Fig. 2 Differences in Soret Band Wavelengths of Gn Fe-TCPP (n = 1-4)

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Peroxidative Activity of Dendritic Porphyrins: The oxidation of 2,6-dimethoxyphenol (DMP) and 3,4-dimethoxybenzyl (veratryl) alcohol (VA) was attempted using free Fe-TCPP and Gn Fe-TCPP (n = 1-4). All the catalysts had the ability to oxidize both phenolic and nonphenolic substrates upon the addition of *m*-chloroperoxybenzoic acid (mCPBA) as an oxidizing substrate. However, the rate of oxidation was fastest with G4 Fe-TCPP; 40 fold faster for DMP and 10 fold for VA compared to free Fe-TCPP. G3 and G4 Fe-TCPPs exhibited typical enzymatic catalysis when the initial rates were plotted against substrate concentrations (Lineweaver-Burk plot, data not shown). On the other hand, free Fe-TCPP and G1 and G2 Fe-TCPPs did not show good correlations for the Lineweaver-Burk plot. The kinetic data suggests that the interface was formed with G3 and G4 dendrons, in other words, Fe-TCPP was encapsulated with G3 and G4 dendrons, but not with G1 and G2 dendrons.

Stability of Dendritic Porphyrins against Peroxide: Free Fe-TCPP could be a catalyst; however, it is easily bleached by mCPBA. The stability against mCPBA was examined by following a decrease at the Soret absorption upon the addition of excess mCPBA in the absence of a reducing substrate. As shown in Fig. 3, G4 Fe-TCPP was extremely stable against 1,000 eq mCPBA (equivalent to Gn Fe-TCPP). After 1,000 eq of reducing substrate was completely oxidized by 500 eq of mCPBA, G4 Fe-TCPP was recovered with no damage (confirmed by NMR, data not shown). Therefore, G4 Fe-TCPP is very stable during the peroxidative catalytic cycle.

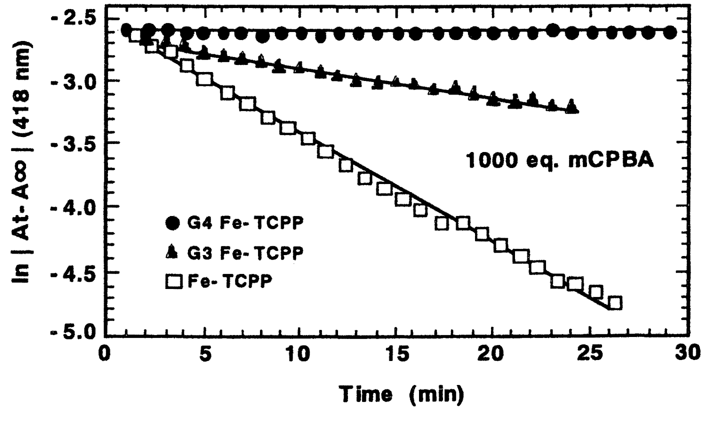

Fig. 3 Effect of Dendron Size on Stability against mCPBA

Coordination of Imidazole Derivatives: The heme iron of peroxidases is usually penta-cooridinated, four nitrogens from heme and one imidazole nitrogen from histidine. This histidine is known as a proximal His and plays an

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important role in the electron push-pull mechanism of the peroxidative catalytic action (15). G4 Fe-TCPP exhibited a peroxidative activity by itself, but a better activity was expected if an imidazole is coordinated properly. Difference spectra were taken and _A data were utilized for the double reciprocal and Hill plot analyses. Ten imidazole derivatives were examined and 2- and 4-methyl imidazoles were found to coordinate well to Fe^{III} in G3 and G4 Fe-TCPPs. The dissociation constants and number of bound molecules were calculated using a double reciprocal and a Hill plot, respectively. Although the bis-form was obtained upon the addition of imidazole derivatives to free Fe-TCPP (data not shown), only one imidazole was coordinated to G3 and G4 Fe-TCPPs (Table 1). Actually, the peroxidative activity of G4 Fe-TCPP was raised by more than 500 fold by adding 4-methyl imidazole (Table 2). The bis-imidazole heme complex has been known as not showing any peroxidative activity, supporting that only one imidazole was ligated to the iron of G4 Fe-TCPP. To better understand the molecular mechanism, the structures of Gn Fe-TCPP were studied using computational chemistry.

Table 1 Interaction of Iron with Imidazole Derivatives

		K_D (mM)	No. of Bound Molecules
G3 Fe-TCPP	imidazole	1.75	1.07
	4-methyl imidazole	0.45	1.35
G4 Fe-TCPP	imidazole	4.38	1.17
	4-methyl imidazole	1.06	1.02

Table 2 Effect of Imidazole Derivatives on G4 Fe-TCPP Activity

Imidazole Derivatives	kcat (min^{-1})	Relative Activity
None	0.0825	1.0
Imidazole	15.6	188.5
N-methyl imidazole	3.24	39.3
2-methyl imidazole	31.3	379.4
4-methyl imidazole	46.3	561.2

Computational Chemistry: The optimized structure of G4 Fe-TCPP was obtained in terms of computational chemistry with molecular mechanics and molecular dynamics. These optimizations were calculated using an MM3 force field. The output of molecular modeling indicated that the most stable structure contained all the dendrons distributed onto one side of the porphyrin plane (Fig. 4). The structure/function relationship was thus studied. The complexes which exhibited an enzymatic activity (G3 and G4 Fe-TCPPs) had asymmetrical structures. On the other hand, in G1 and G2 Fe-TCPPs which were collision type catalysts, the iron was exposed to the solvent. This result may explain that only one imidazole was coordinated to G3 and G4 Fe-TCPPs but bis coordination was observed with the dendritic porphyrin with smaller or no dendrons. Asymmetrical distribution of dendrons was suggested from the calculation. This may suggest a different environment for each side of the porphyrin (Fig. 4). The steric effect caused by larger dendrons was also observed in the inactivation study (Fig. 3). Further study is currently under way.

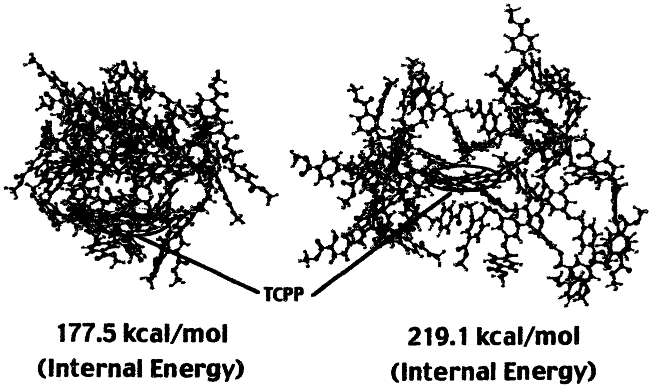

Fig. 4 Optimized Structure of G4 Fe-TCPP

Role of Imidazole: During the oxidation of DMP and VA, not only one electron abstraction but also one proton transfer occurred. (Note: Products are quinone dimer (16) and veratraldehyde (4, 5), respectively). The next issue was to locate the destination of the proton. In the peroxidase reaction, the distal His plays a role to uptake the proton released from a reducing substrate, then which is released with ferryl oxygen as water (15). However, G3 and G4 Fe-TCPPs do not possess a distal imidazole or any equivalents. Fig. 5 shows the relationship between the peroxidative activities and water contents in the solvents, indicating that the less the water, the higher the activity. This result indicated that water might not be a proton acceptor in this system. Probably, the imidazole compounds may uptake protons released during the reaction.

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Effect of Solvents: It is still unclear what properties of organic solvents would affect the enzymatic activity. Since the solvent's hydrophobicity (expressed by the log *P* value) is known to be one of useful parameters in predicting the degree of enzymatic activity in anhydrous organic media (17), the oxidation of DMP by G3 and G4 Fe-TCPPs was carried out in various organic solvents covering a wide range of hydrophobicity (Fig. 5). Although no strong correlation exists between enzymatic activity and the log *P* value of the solvents, the complex generally exhibited a high catalytic activity in hydrophobic solvents. The complexes did not show activity in hydrophilic solvents. One possible reason is the solubility of reducing substrate and/or imidazole derivatives in the solvents.

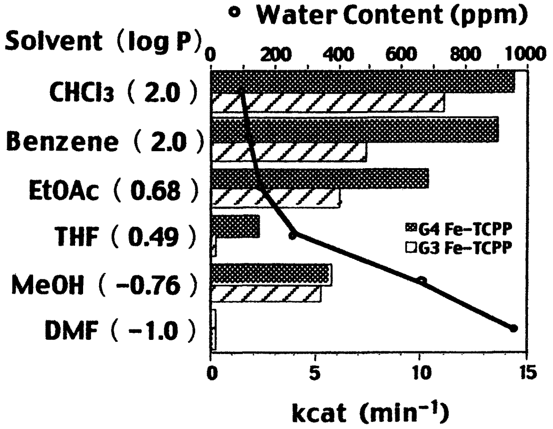

Fig. 5 Effect of Solvents and Water Contents on Activities

Catalytic Performance of G4 Fe-TCPP: After optimizing the reaction conditions, G4 Fe-TCPP exhibited high and stable peroxidative activities. When mCPBA and DMP were utilized as oxidizing and reducing substrates, respectively, the value of kcat was found to be about 10^4 M^1s^{-1}. In the view of this kinetic data and the stability of the complex (intact after 500 catalytic rotations), one may classify G4 Fe-TCPP as an artificial peroxidase.

AKNOWLEDGMENT

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Chapter 14

A Manganese-Based Catalyst for Alkaline Peroxide Bleaching

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The manganese complex [Mn(III)$(\mu\text{–O})_2(\mu\text{–CH}_3\text{-COO})$Mn(IV)$L^2$]$(ClO_4)_2$, where L^2 = 1,2-bis-(4,7-dimethyl-1,4,7-triazacyclono-1-yl)ethane, is shown to act as a catalyst (10-20 ppm) in hydrogen peroxide bleaching stages. The incorporation of this catalyst in a bleach stage results in fast hydrogen peroxide consumption at low temperatures. Consequently, the higher peroxide consumption improves delignification in a more selective manner than the delignification obtained by more vigorous reaction conditions in uncatalyzed bleaching stages. Model compound experiments showed that the catalyst assisted hydrogen peroxide system (P_{cat}) is able to degrade residual lignin structures, which are not degradable by hydrogen peroxide alone. The application of the catalyst in peroxide bleaching gives better strength properties, lower COD loads, and higher yields. The catalyzed peroxide system has been examined in different TCF bleaching sequences for various pulps. Catalyst assisted bleaching made possible the bleaching of softwood kraft pulps to high brightness in short sequences, i.e. OQ(OP)P. Such sequences based only on oxygen and hydrogen peroxide facilitate countercurrent washing and reduce the consumption of fresh water. Investigations on the stability of the catalyst show that suitable storage and mixing conditions must be maintained. To date the catalyst is not available commercially and there is no information about the cost of this complex.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Introduction

Manganese complexes play an important role in biochemical processes. Examples are the oxidation of water via photosynthesis to oxygen, the disproportionation of hydrogen peroxide by dismutase, the participation of Mn(II) in metal ATP complexes, and the reduction of ribonucleotides in some bacteria *(1)*. For a better understanding, model compounds with well-understood structural and electronic configurations mimicking the active sites of the enzymes participating in the aforementioned processes were synthesized *(2)*. The chemical behavior of manganese complexes toward hydrogen peroxide attracted not only researchers dealing with basic research, but also those engaged in the field of applied research. Hage and coworkers *(3)* published results of some of these complexes being applied as catalysts in bleaching of textiles with hydrogen peroxide. They clearly demonstrated that the catalyst assisted bleaching treatments and improved the removal of tea stains from clothes. The research and development of the detergent industry is driven by a trend to decrease washing temperatures. This necessitates the activation of the bleaching chemicals. Among the transitional metal ion complexes, iron and manganese complexes are most suitable for this purpose and have become the focus of research for such applications. The most promising complexes, which Hage et al. described, consist of a ligand system derived from 1,4,7-triazacyclononane (Figure 1). Complexes of this type were also used as catalysts for selective epoxidation of olefins *(4,5)*, oxidation of benzylalcohols to benzaldehydes *(6)*, and oxidation of different phenols *(7)*.

A similar tendency, as in the detergent industry, can be seen in the research and development of TCF bleaching of pulp, in which efforts have been focused to increase the reactivity of hydrogen peroxide and oxygen to residual lignin in pulp. Since tea stains in clothes are mostly caused by polyphenolic tannins, their removal from clothes is very similar to the removal of lignin, also a polyphenolic compound, from pulp. This was the basic idea behind using manganese complexes of this type as catalysts in hydrogen peroxide bleaching of pulps *(8-16)*. In addition, the reaction mechanism for delignification in the manganese complex-catalyzed peroxide bleaching stages was elucidated by studying the behavior of the catalyst towards lignin model compounds and the structural changes of residual lignin in the catalyzed bleaching (*17*-20). In this paper, we summarize the knowledge concerning the use of these catalysts in peroxide bleaching of different pulp grades.

Selection of Catalyst

The pH dependence of the bleaching activation by three of the most promising complexes that has been tested in textile bleaching shows distinct

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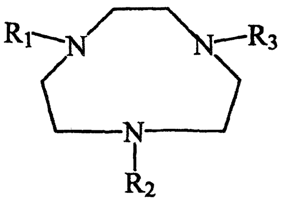
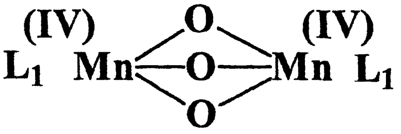


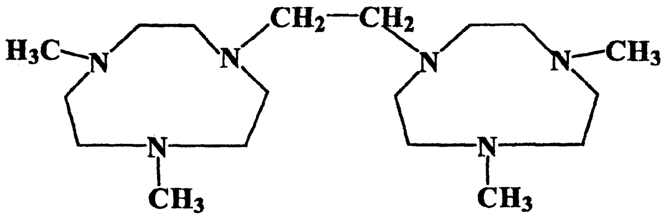


L_1: $R_1 = R_2 = R_3 = CH_3$

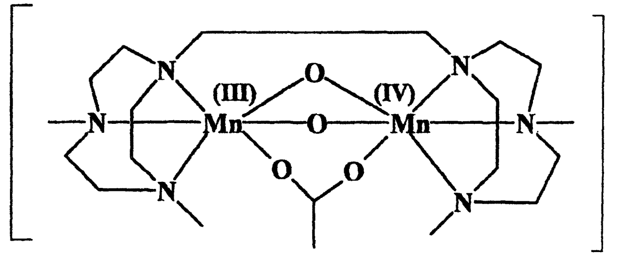
L_2: $R_1 = R_2 = R_3 = CH_2CH_2OH$

K1

L_3:



K2:



$(PF_6)_2$

Figure 1. Ligands and core structures
L_3 = 1,2-bis-(4,7-dimethyl-1,4,7-triazacyclonon-1-yl)ethane

differences (Figure 2). The complexes K1 and K3 have a higher potential to activate bleaching reactions at pH values less than 10 as compared to complex K2. However, the complex K2 seems to be more alkaline stable showing an even better activation at pH values higher than 10. A consequence of the presumed differences in alkaline stability is a difference in optimal pH value for maximal peroxide activation. The complexes K1, $[Mn(IV)_2\text{-}(\mu\text{-}O)_3L^1](PF_6)_2$, where L^1 = 1,4,7-trimethyl-1,4,7-triazacyclononane and K2 $[Mn(III)(\mu\text{-}O)_2\text{-}(\mu\text{-}CH_3\text{-}COO)(Mn(IV)L^2](ClO_4)_2$ with L^2 = 1,2-bis-(4,7-dimethyl-1,4,7-triazacyclonon-1-yl)-ethane (Figure 1) were chosen for the pulp bleaching experiments. There they show a nearly identical pH dependence compared to the textile bleaching experiments in Figure 2. The complex K2, however, shows a much better selectivity in terms of delignification or brightness versus viscosity. For these reasons K2 was selected as the catalyst in the peroxide bleaching experiments.

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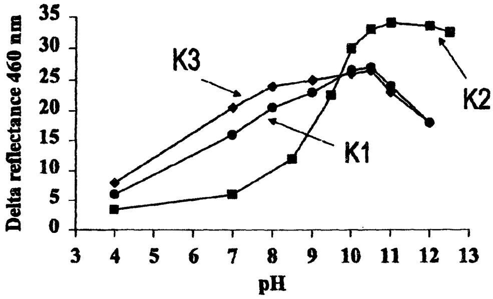

Figure 2. Tea stain bleaching of test clothes
K3: a mononuclear Mn complex
Reproduced with permission from reference (*3*)

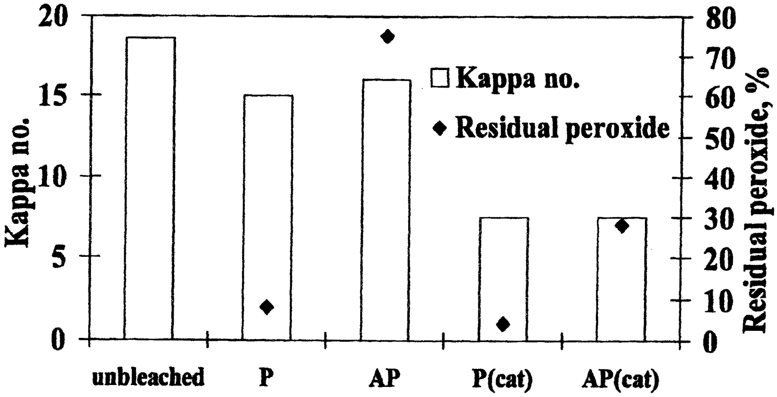

Figure 3. Delignification by catalyst assisted hydrogen peroxide bleaching (*8*)
Starting material: unbleached softwood kraft pulp, A = acid wash
Bleaching conditions: 4 % H_2O_2, 7.5 % NaOH, 70 ppm cat, 50 °C, 3 % cons. 75 min

Effects of Catalyst Activation in Hydrogen Peroxide Bleaching

The application of 70 ppm of K2 to a peroxide stage shows a much better delignification than a conventional peroxide stage (Figure 3). The bleaching conditions, however, were adjusted in the peroxide stage to see the effects of the catalyst addition. To avoid mixing problems, a low pulp consistency was applied. High chemical charges were chosen to elaborate the effects of the catalyst addition. Also, the application of the catalyst on unbleached softwood kraft pulp is not recommended. The high phenolic hydroxyl group content in the residual lignin of unbleached pulps gives rise to a good oxygen delignification and there is no need for more expensive bleaching chemicals. The hydrogen peroxide could nearly be fully consumed resulting in a much better lignin removal. The high peroxide consumption in the trial without catalyst input could be attributed to the peroxide degradation via transition metal ions. The trial with an acid washed pulp resulted in a much lower peroxide consumption and a slightly lower delignification, which indicates the well known fact that the formation of hydroxyl radicals is responsible for an appreciable part of the lignin removal in hydrogen peroxide stages. The catalyzed trial with the acid washed pulp gave also a lower peroxide consumption indicating that in the case of catalyst application a part of the peroxide is also unspecifically consumed by the catalytic action of transition metal ions. The higher peroxide portion could however not be used for further delignification. A reason could be that all lignin structures capable of reacting with the catalyzed system are consumed.

In trials with acid-washed oxygen-delignified softwood kraft pulp (AO) important parameters in catalyst assisted peroxide bleaching were investigated. With higher NaOH charges the gap in delignification between catalyzed and uncatalyzed trials becomes larger (Figure 4). The peroxide consumption is more sensitive to changes in pH than is the delignification . Here, a drastic increase in the peroxide consumption can be observed when the pH of the bleaching liquor containing the catalyst exceeds a value of 10-11. The high activation potential of K2 at high pH values may be partly explained by the relatively high stability of the binuclear core. This was shown by a more stable 16 line signal in the electron spin resonance (EPR) spectra of K2 at pH 12 compared to K1 (*3*). The 16 line spectra is typical for mixed valence Mn(III)Mn(IV) complexes. This behavior is consistent with the results of textile bleaching (Figure 2). A series of bleaching experiments at varying reaction times reveals that the catalyst assisted bleaching causes a very rapid peroxide consumption (Figure 5). Within the first 15 minutes most of the peroxide has been consumed indicating that the small further delignification must be ascribed to leaching effects. The catalytic activation renders possible not only lower reaction temperatures but also much shorter reaction times.

A general overview of the results from these early trials shows a much better selectivity in favor of the catalyzed bleaching stages (Figure 6). However, the bleaching conditions were advantageous for the catalyzed trials, especially, because of the low reaction temperature of 50°C applied in these experiments. It is well known that hydrogen peroxide as a delignifying agent needs reaction temperatures higher than 50°C, but in the uncatalyzed bleaching stages the

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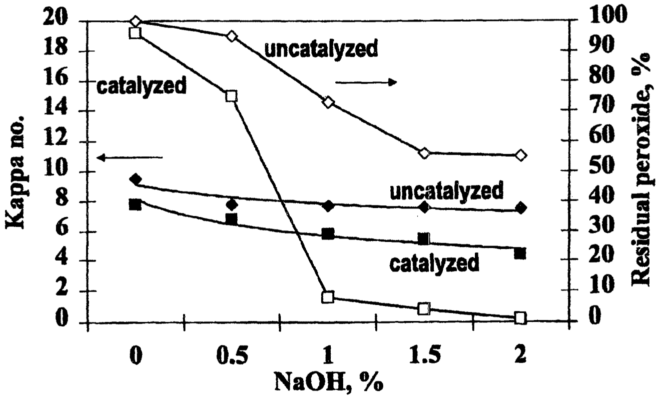

Figure 4. Influence of alkali charge in catalyst assisted hydrogen peroxide bleaching (8)

Starting material: OA pretreated spruce kraft pulp

Bleaching conditions: 2 % H_2O_2, 60 ppm cat, 50 °C, 10 % cons, 120 min

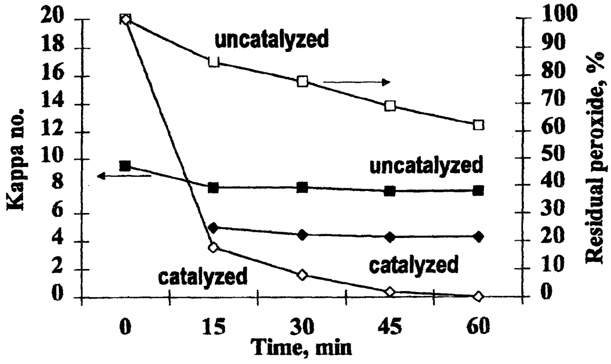

Figure 5. Influence of retention time in catalyst assisted hydrogen peroxide bleaching (8)

Starting material and bleaching conditions as described in Figure 4 carbohydrates are further degraded also at the relatively low reaction temperature of 50 °C. This leads partly to the much better selectivity of catalyst-assisted hydrogen peroxide bleaching. The improved selectivity of catalyst assisted bleaching is also demonstrated in the final TCF bleaching stage applied to a softwood kraft pulp. Results of this investigation are discussed below .

In these first sets of bleaching experiments the application of this manganese-based catalyst revealed some basic trends. The catalyst contributes to a very fast peroxide consumption resulting in a higher delignification with a better selectivity. These effects can be accomplished at a temperature as low as 50°C, which emphasizes the high activation potential of this catalyst.

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Lignin Models and supposed Reaction Mechanism

Experiments with lignin model compounds show that the catalyst enables the degradation of structures which cannot be degraded by hydrogen peroxide alone (Figure 7). Oxidation of α-hydroxyl groups to ketones and the epoxidation of conjugated C=C bonds have been demonstrated in several publications (*17-19*). The time dependence of the catalytic degradation is similar to pulp bleaching. In both cases, the reaction is very fast.

Regarding the reaction mechanism, Cui et al. gave a hypothesis that K2 is activated in the presence of hydrogen peroxide by the cleavage of the acetate bridge and changing it to a third oxo-bridge (Figure 8). An oxidation of the Mn(III)Mn(IV) to Mn(IV)Mn(IV) takes place during the activation. Thus, the so-called precatalyst is transformed to the active catalyst (Figure 8). In this form the catalyst is participating in a catalytic cycle (Figure 9). At first, one of the oxo-bridges of the catalyst core structure is cleaved by nucleophilic attack of a hydroperoxide anion on one of the Mn(IV) nuclei. The next step is a single-electron-transferring oxidation of the substrate to produce either a corresponding phenoxyl radical or aryl cation radical depending on the nature of substrate. In a further step a hydroxyl radical is released from the catalyst, which reacts at once with the partly oxidized substrate. The authors claim this concerted action as one explanation for the high selectivity of K2.

The supposed reaction mechanism is supported by EPR experiments, where the addition of oxidant to K2 yields a silent EPR signal indicating a Mn(IV)Mn(IV) state. A further indication for the activation of the precatalyst is the fact that K2 is not able to oxidize catechol without the addition of oxidant. This contrasts with K1, where the Mn(IV)Mn(IV) state is originally present (3).

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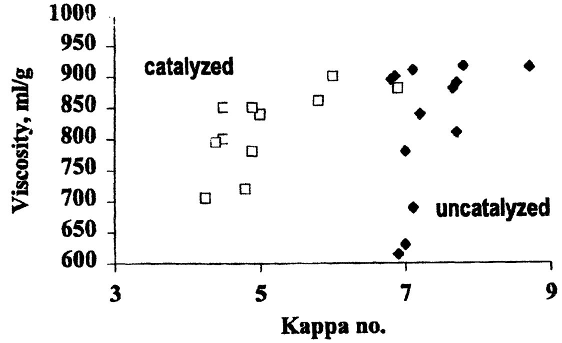

Figure 6. Selectivity of P stages of unbleached pulp (8)
Starting material: OA pretreated spruce kraft pulp
Bleaching conditions: 1-4 % H_2O_2, 0-3 % NaOH, 60 ppm cat, 50 °C, 10 % cons, 120 min

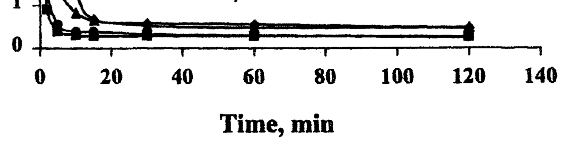

Figure 7. Decrease of 1-(3,4-Dimethoxy-phenyl)-1-propene (17)

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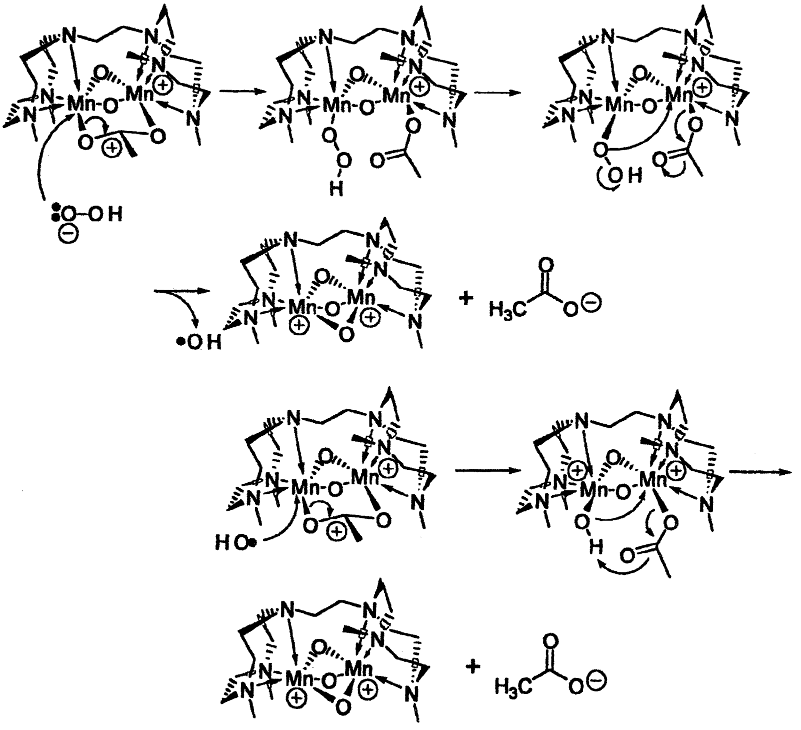
$$HO{-}OH \xrightarrow[\text{[HO}^-\text{]}]{} HO{-}O^{\ominus} + H^{\oplus}$$



Overall Reaction

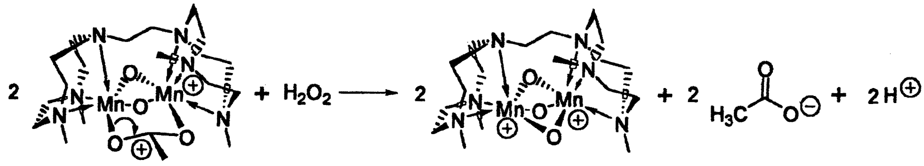


Figure 8. Transformation of the precatalyst to the active catalyst (*17*)

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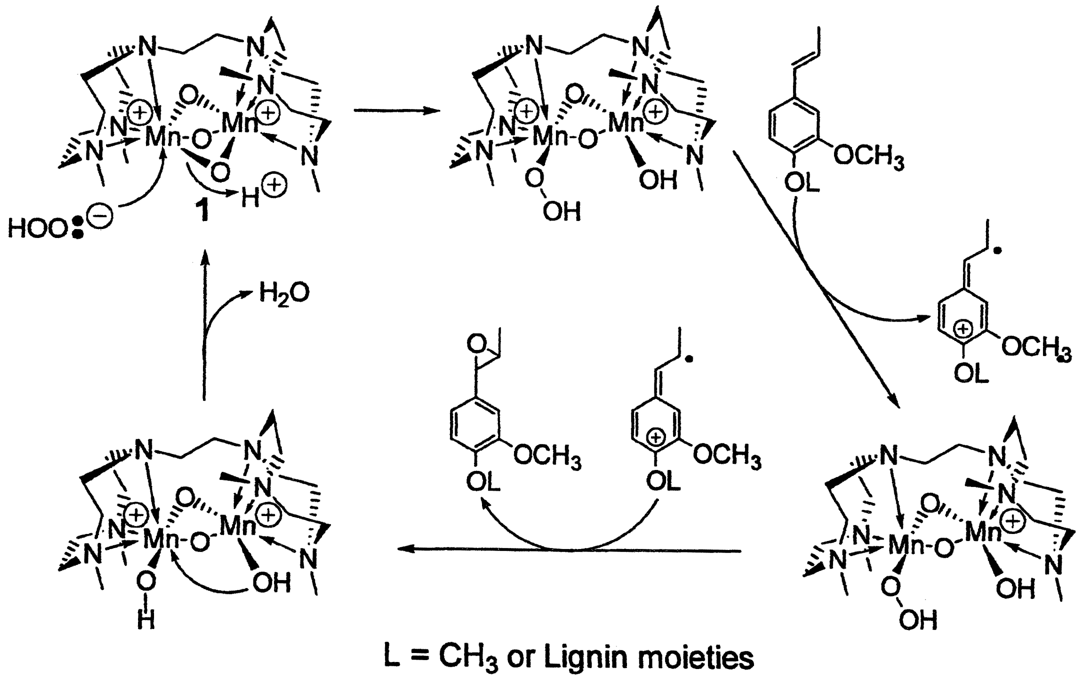
HOO$^{\ominus}$
1
$H^{\oplus}$
H_2O
OCH$_3$
OL
L = CH$_3$ or Lignin moieties

Figure 9. Catalytic cycle of activated K2 (20)

In oxidation experiments of phenolic compounds with K1 and H_2O_2, phenoxyl radicals could be detected indicating the oxidation of phenolic substrates via single-electron-transferring steps (7). Epoxidation reactions with a similar catalyst and $H_2{}^{18}O_2$ showed a product with 100% ^{18}O suggesting that H_2O_2 and not water is the oxygen source (3), which is also in keeping with the proposed reaction mechanism.

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Catalyzed P stages in TCF sequences

The examples above show the effects of catalyst application in the bleaching of unbleached and oxygen delignified pulp. In both cases, the improvements by using the catalyst can be ascribed to the enhanced delignification. Results in reference (8) show that brightness gains can also be achieved by using the catalyst in final peroxide bleaching of A(OP)Z-treated pulps. At low ozone input in the Z-stage the brightness gain by using the catalyst in the P-stage is approximately 10 points at 50°C and approximately 5 points at 90°C. Brightness gains attributable to catalyst–assisted P-stage bleaching has been observed even on pulps with a low lignin content of approximately 0.3 %, obtained by using high ozone charges in Z.

In high brightness TCF bleaching (brightness >88 % ISO), Z stages are often necessary to reduce the lignin content before final peroxide bleaching. The carbohydrate degradation by ozone is difficult to control. To confine the degradation of carbohydrates in Z stages, low reaction temperatures and low pH values are required. However, these conditions do not fit very well in the temperature and pH profile of a whole sequence. Therefore, it was investigated if the delignification enhancement of the catalyst can lead to a high final brightness with only oxygen and peroxide as the active bleaching chemicals. Patt and Mielisch (*10*) demonstrated the bleaching of a softwood kraft pulp to high brightness levels in very short sequences (OQ(OP)P). TCF sequences based only on oxygen and peroxide have the advantage of a more homogenous pH and temperature profile facilitating a further closure of the water loops in bleaching lines, which can enable further reduction in water consumption. Prices for the catalyst are not known yet, but it seems clear that the catalyst charge must be below 20 ppm and applied in a single application to be economical . Such constraints indicate that it is more favorable to use the catalyst in the final P stage instead of in the (OP) stage at the beginning of the sequence (*10*).

An example of short-sequence bleaching of softwood pulps was demonstrated on an unbleached softwood ASAM pulp. After a chelation stage to remove transition metals, the pulp was bleached in only one $(OP)_{cat}$ stage to a brightness of 85 % ISO whereas the conventional (OP)-stage bleached the pulp to a brightness of 75.8 % ISO. This is possible because of the easy bleachability of this type of pulp. The high viscosity of the unbleached pulp and the high selectivity of the (OP) stage resulted in a bleached pulp viscosity of over 1000 ml/g. Consequently very high strength properties were measured. The breaking length of this bleached pulp was above 12 km (*9*).

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Catalyzed (OP) Stages compared to $(OP)_{HT}$

A catalyst-assisted (OP)-stage operated at low temperature (50 °C) can be an attractive alternative to a high temperature pressurized (OP)-stage frequently used as a final bleaching stage in TCF sequences. Therefore, Kühne et al. (*12*) compared $(OP)_{HT}$ and $(OP)_{cat}$ (Figures 10-12). The reaction time of the catalyzed trials was halved because of the faster peroxide consumption compared to the control . A catalyst charge of 20 ppm on pulp in (OP) resulted in a brightness difference of approximately 5 % ISO . This brightness gain can be fully compensated for by higher reaction temperatures in a conventional (OP)-stage. However, at the same brightness level, the application of catalyst results in pulps with approximately 80 ml/g higher viscosity(Figure 11).

The increase of the catalyst charge to more than 10 ppm on pulp results in only marginal further brightness improvements. Under the conditions employed a catalyst charge higher than 10 ppm is not justified. There is even the possibility that less than 10 ppm on pulp will result in clear brightness improvements. The best catalyst charge must be surely checked in each application case. The catalyst input makes possible temperature and time reduction yielding the same brightness levels. The higher selectivity gives rise to better strength properties (Figure 12). The lower degradation of carbohydrates can be traced back to the smoother conditions in the catalyzed peroxide stages with respect to reaction temperature and time. These benefits give higher bleached yields and lower COD loads (*21*).

Other pulp types

Different publications deal with catalyst-assisted bleaching of pulp types other than softwood kraft pulps (9,*11*,*16*). The application of catalyzed peroxide bleaching to a hardwood kraft pulp showed somewhat different results compared to the bleaching of softwood kraft pulp. With an increasing charge of catalyst in a peroxide treatment of an OQ-delignified eucalyptus pulp, a

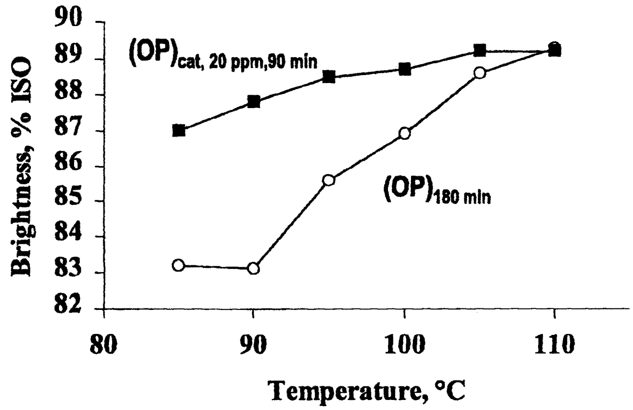

Figure 10. Influence of temperature in catalyst assisted final (OP) stages (12)

Starting material: OQ(OP)Q pretreated softwood kraft pulp

Bleaching conditions: Catalyzed / uncatalyzed - 90 / 180 min, 3.5 % H_2O_2, 2.5 % NaOH, 0.05 % DTPMPA, 0.2 % $MgSO_4$

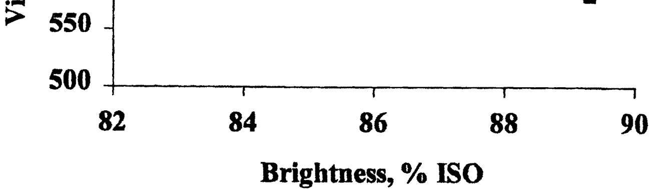

Figure 11. Selectivity of final $(OP)_{cat}$ (12)

Starting material and bleaching conditions as described in Figure 10

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brightness gain of about 8 % ISO can be observed at 50°C (Figure 13). Higher catalyst charges result in only minor brightness increases. The brightness gains by catalyst application can compensate for more vigorous bleaching conditions in a conventional P-stage with respect to reaction temperature, reaction time, and chemical input. For example, the effect of a catalyst charge of 10 ppm on pulp allows the reaction time to be halved. At high reaction temperatures there is only a small brightness gain, which, however, does not justify the additional catalyst input. Also in the case of hardwood pulp bleaching there are effects that can be attributed to the catalytic assistance. But these effects can be nearly fully compensated for by more vigorous bleaching conditions. Catalyzed peroxide stages seem to be more useful for softwood pulp bleaching. High brightness levels in hardwood pulp bleaching are easier to achieve, which makes the application of the catalyst not necessary.

In contrast to the results from the bleaching of eucalyptus kraft pulp, improvements in bleaching can be achieved by using the catalyst on a prehydrolyzed eucalyptus kraft pulp which require a very high purity when used as a bleached dissolving grade pulp. (Table 1) The following specifications were set for production of a TCF dissolving pulp: kappa number less than 0.4, brightness higher than 92 % ISO and a viscosity higher than 600 ml/g. In the TCF sequence applied, the required kappa number and brightness could be met by adjusting the ozone charge but the viscosity losses were too high (Table I). All the specifications could be met only by using an $(OP)_{cat}$-stage due to its more selective delignification compared to a conventional (OP)-stage.

In catalyzed peroxide bleaching trials with Mg-sulfite pulp, brightness gains in favor of catalyzed bleaching stages of approximately 2 points could be obtained (*9*). Here similar arguments can be made as in the bleaching of hardwood pulps. Sulfite pulps are also relatively easy to bleach and therefore the benefits from catalyst assisted bleaching are smaller than in bleaching of softwood kraft pulps.

In peroxide bleaching of wood free waste paper pulp, clear benefits can be derived from the catalyst application in terms of brightness and delignification (Figure 14). At a catalyst charge of 20 ppm the brightness increased by 3 units and the kappa number decreased by 4 units. This process is applicable to wood-free waste paper pulps with a low lignin content, it is unlikely that the catalyst would improve peroxide bleaching of high-lignin recycled pulps.

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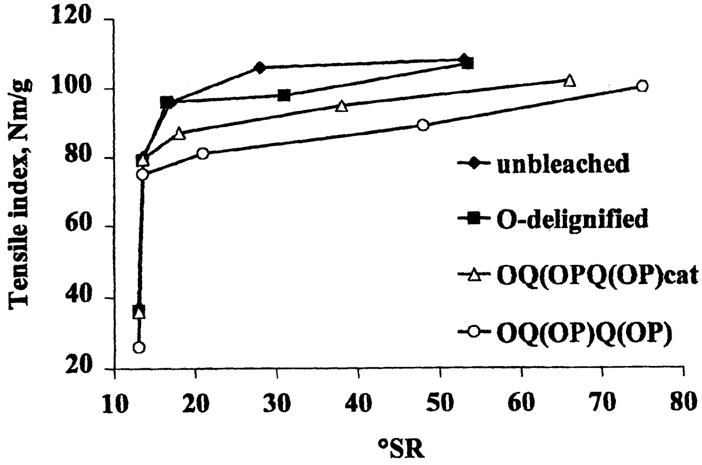

Figure 12. Comparison of pulp strength of $(OP)_{HT}$ versus $(OP)_{cat}$ (12) Starting material and bleaching conditions as described in Figure 10

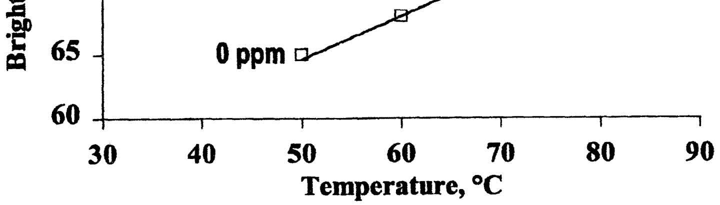

Figure 13. Catalyst assisted bleaching of eucalyptus kraft pulp (11) Bleaching conditions: Catalyzed / uncatalyzed - 90 / 180 min, 3.5 % H_2O_2, 2.5 % NaOH, 0.05 % DTPMPA, 0.2 % $MgSO_4$

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Table I. Comparison between catalyzed and uncatalyzed bleaching of OQ pretreated prehydrolyzed eucalyptus kraft pulp

Stage	Kappa no.	Brightness (% ISO)	Viscosity (ml/g)
Q	-	-	-
OP_{Cat}	2.7	75	786
$Z_{0.3\%}$	1.2	86.1	632
Q	-	-	-
P2	**0.4**	**93.4**	**578**
Q	-	-	-
OP	4.1	66.9	859
$Z_{0.3\%}$	2	77.4	647
Q	-	-	-
P2	**0.9**	**88.3**	**604**

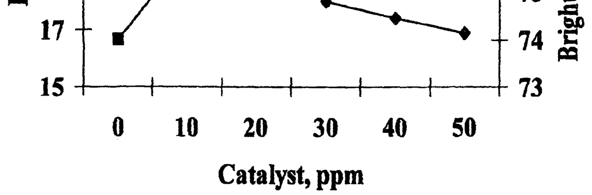

Figure 14. Catalyst assisted bleaching of waste paper pulp (wood free DIP) (16)

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Catalyst Stability

For an industrial application, the stability of the catalyst in terms of activation potential must be known. This holds true especially for conditions, which can be expected in pulp mills, for example during mixing operations. This was investigated in a two-stage treatment (Figure 15). In the pretreatment step the catalyst was exposed to various pH and temperature conditions. The pretreated catalyst was subsequently used in a standard P stage. The aim was to see what conditions are most detrimental for the catalyst activity. At pH values higher than 11.5 in the pretreatment step, a drastic increase in the residual peroxide of the following standard P stage can be seen with increasing pH values (Figure 15). The lower peroxide consumption corresponds to a loss in brightness to a level, which is at pH 13 and 80 °C similar to a reference peroxide bleaching stage without catalyst input. As expected, the losses in bleaching activity at pH values higher than 11.5 are more pronounced at higher temperatures (Figure 16). A more detailed investigation showed that at a pH value of 11.5 in the pretreatment, the catalytic effect in the following P stage is getting smaller with longer pretreatment times. This effect can not only be seen by higher residual peroxide contents in the standard P stage but also in a lower brightness. In the case of pH 12 at 120 minutes (*14*), the brightness after the P stage goes down to the level of a reference without any catalyst input indicating a total loss of activation potential. As a consequence, storage and mixing conditions of the catalyst must be exactly maintained to avoid unwanted losses in activation potential. Under laboratory conditions no losses in activation potential of the aqueous solutions of the catalyst could be observed.

Investigations dealing with the influence of different substituents on the 1,4,7-triazacyclononane structure, different solvent systems and different oxidants/additives in catalyzed oxidations (22, 5, 6) show possibilities for further improvements. For example, the addition of acetic acid increases the turnover number in the oxidation of hexane from 86 to 1350 (*22*). Future investigations will show whether further improvements can be achieved in bleaching of pulps with hydrogen peroxide using this type of catalysts. The main target of actual research is to find a new synthesis pathway to reduce the production costs of the catalyst K2.

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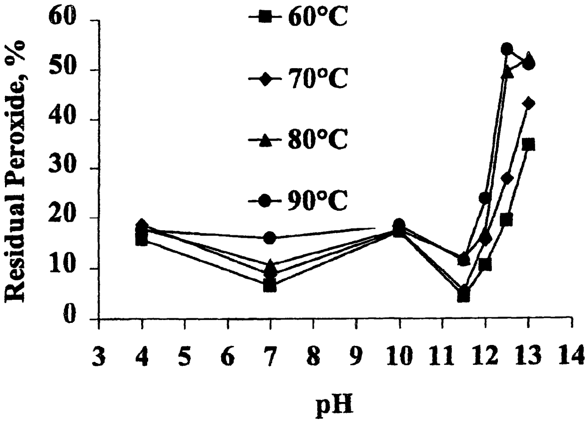

Figure 15. Influence of pH in pretreatment on stability of catalyst - residual peroxide after standard P stage (14)
Pretreatment conditions: pH = 4 – 13, 60 – 90 °C, 10 min
Standard P stage: 2 % H_2O_2, 0,75 % NaOH,60 °C, 60 min, 0.2 % $MgSO_4$

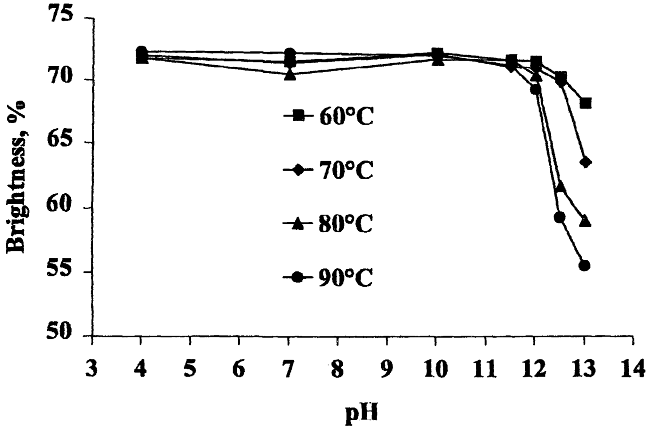

Figure 16. Influence of pH on stability of catalyst – brightness (14)
Conditions as described in Figure 15.

(III) and -(IV) of Biological Relevance. The Crystal Structure of $[L'Mn^{IV}(\mu\text{-}O)_3Mn^{IV}L']PF_6)_2\bullet H_2O$ Containing an Unprecedented Short Mn •••Mn Distance of 2.296 Angström. *J. Am. Chem. Soc.* **1988,** *110*, 7398-7411.

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Chapter 15

Involvement of Oxygen-Derived Free Radicals in Chemical and Biochemical Degradation of Lignin

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The formation of superoxide and hydroxyl radicals and their reactions during oxygen delignification are reviewed. It is concluded that:

- Oxygen delignification is a free radical process governed by the interplay between superoxide and the hydroxyl radical.
- Current process conditions favor carbohydrate degradation.
- Hydroxyl radicals may play an important part in the initial phase of microbial wood degradation.
- Oxygen delignification should be carried out under milder conditions: This would require the development of specific oxidation catalysts.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
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State of the Art

Oxygen delignification (bleaching) is a very attractive method to remove lignin from pulp now being implemented in most modern pulp mills. The main advantage of this process is good economy and little environmental impact. However, under current conditions, *i.e.* high pH and a temperature of ~ 100 °C, only uncondensed phenolic units and olefinic structures, like stilbenes and enol ethers, are rapidly degraded (*1,2,3*). Condensed phenolics such as 5-5′ biphenolic structures that originate from protolignin, or arise via radical coupling reactions, are fairly resistant (*3*) and dominate the condensed phenolic units in residual lignin (*4*). Diphenylmethane structures, formed during kraft pulping, are virtually unaffected by oxygen treatments. The same applies to non-phenolic β-aryl ether structures. Therefore, lignin elimination would be considerably improved if these resistant structures could be degraded. However, attempts to further delignify the pulp by treatment with oxygen are rendered difficult due to slow kinetics and/or to excessive degradation of the polysaccharides with subsequent decrease in pulp strength. Evidently, both the efficiency and selectivity of oxygen delignification must be improved in order to make full use of its potential. Recently, by introducing a new two-stage procedure including peroxide addition (*5*), some advances in this direction have been made.

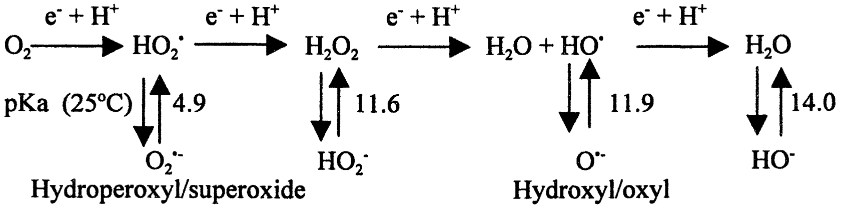
Basic Chemistry of Oxygen Delignification

The Initial Reaction

The ultimate driving force of oxygen delignification is the four-electron reduction of molecular oxygen to water:

$$O_2 + 4e^- + 4H^+ \longrightarrow 2\,H_2O$$

This reaction is strongly exothermic and would, if realized, allow oxidation of all lignin components of pulp. However, molecular oxygen is a triplet in the ground state (T_0). Therefore, in the uncatalyzed reduction of oxygen, mechanistic rationale precludes utilization of this energy in a primary electron transfer step. Instead, the oxygen reduction proceeds in four consecutive one-electron steps (*6*):



Biological oxidation by O_2, catalyzed by specific enzymes, probably occurs by an oxygen-atom transfer mechanism (oxygenases) or by sequential one-electron transfer steps with concerted stabilization of intermediates (cytochromes). The oxygen-atom transfer mechanism requires a close association between substrate and enzyme, whereas oxidation involving a cytochrome type of enzyme is less substrate dependent. Presumably, lignin-degrading enzymes belong to this second category. In the uncatalyzed oxygen delignification, the initial step is a one-electron transfer from the substrate (pulp) to oxygen. The standard one-electron reduction potential of O_2 is –0,33V vs NHE (*7*) and is independent of pH above pH 5. Hence, this initial step is not energetically favorable and proceeds at a reasonable rate only with easily oxidized substrates. Oxidation potentials plotted *vs*. pH for some lignin model compounds (*8*) are shown below:

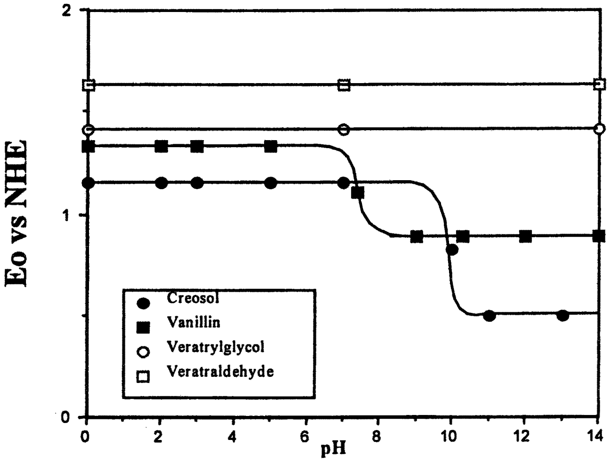


Figure 1. Oxidation potentials for some lignin model compounds plotted vs. pH.

Evidently, the oxidation potentials of phenolates are significantly lower than those of undissociated phenols and phenol ethers. For this reason, oxygen delignification is usually performed under alkaline conditions and the most probable initial step is an outer-sphere one-electron transfer from phenolate to oxygen:

$$PhO^{-} + O_2 \rightleftharpoons [PhO^{\bullet} + O_2^{\bullet -}] \longrightarrow PhO^{\bullet} + O_2^{\bullet -}$$

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This would result in an initial triplet pair consisting of a phenoxyl radical and superoxide in a solvent cage. Apart from the back reaction, which is much favored due to the large difference in the reduction potentials for the redox couples $PhO\bullet/PhO^-$ and $O_2/\bullet O_2^-$, we have not been able to demonstrate any specific chemical significance of the solvent cage. Thus, it should be possible to trace all reactions leading to oxygen delignification back to the initial formation of phenoxyl radicals and superoxide.

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Superoxide

Superoxide, $O_2^{\bullet -}$, and its protonated counterpart, the hydroperoxyl radical, $HO_2^{\bullet}$, react together at almost diffusion-controlled rate (*9*) by dismutation, giving oxygen and hydrogen peroxide. In contrast, the rate of the direct dismutation of superoxide is negligible. In practice, protons or metal ion complexes always catalyze this reaction. Above pH 6, the rate of the proton-catalyzed dismutation can be expressed by the equation: $k = 6\bullet 10^{(12-pH)}\ M^{-1}s^{-1}$ (*9*). Thus, in alkaline solutions, the lifetime of superoxide may be considerable. This means that under the conditions of oxygen delignification, superoxide is diffusible and can *penetrate fibers*. In organic solvents, superoxide is a strong nucleophile; *e.g.* it can release Cl^- from CCl_4. In water, however, superoxide is extensively hydrated and its nucleophilic character is much less pronounced (*10*). Superoxide acts mainly as a reducing agent, but may also act as a weak oxidant towards molecules with readily transferable hydrogen atoms (*11*). In this context, one may consider the following reaction sequence:

$$PhOH + O_2^{\bullet -} \rightleftharpoons [PhO^- + HO_2^{\bullet}] \longrightarrow PhO^{\bullet} + HO_2^-$$

According to this reaction proton transfer from a phenol to superoxide is directly followed by electron transfer to give the corresponding phenoxyl radical and hydrogen peroxide. Using $pK_a = 4.9$ for $HO_2^{\bullet}$ (*9*) and pK_a about 10 for lignin-like phenols (*12*), the equilibrium constant for the proton transfer is of the order of 10^{-5}. In view of this fact, the suggested reaction may seem unlikely. The electron transfer, however, is energetically favorable since the reduction potential of the hydroperoxyl radical is 0.75V (*13*) and the oxidation potential of lignin-related phenolates is about 0.55V (*8*). Given $\lambda^{\circ} = 168\ kJmol^{-1}$ (*14*), we estimate $k \approx 2\,10^5\ M^{-1}s^{-1}$ by the Marcus theory. Hence, the electron transfer may be fast enough to make the overall reaction rate significant. In conventional oxygen delignification, the direct superoxide driven oxidation of phenols does not play an important part because, under prevailing conditions, phenols are deprotonated and the driving force of the reaction is the formation of the reaction pair depicted within the brackets. In peroxide bleaching, on the other hand, this reaction may be important. Experimental support for this is obtained from column displacement bleaching experiments with unbleached pulp (*15*). Unexpectedly, the highest brightness gain was not found at the first but at the second plate. A possible explanation of this result is that hydrogen peroxide at the top level partially decomposes into superoxide which travels down the column with the bleaching liquor and reacts according to the reaction suggested above when pH has fallen below 10. Another example of the concomitant abstraction of a proton and an electron by superoxide is the reaction with dihydroxybenzenes affording semiquinone radical anions (*16*, *17*). Although outside the scope of this review, it is interesting to note that reactions of the suggested type may be involved in the biosynthesis of lignin (*18*).

The Hydroxyl Radical

Reduction of hydrogen peroxide may lead to formation of hydroxyl radicals (*19*). In oxygen delignification, this reduction is achieved mainly by superoxide. However, superoxide does not react directly with hydrogen peroxide at any significant rate (*20*) and in practice this reaction is catalyzed by certain transition metal ions:

$$M^{n+} + O_2^{\bullet -} \rightleftharpoons M^{(n-1)+} + O_2$$

$$M^{(n-1)+} + H_2O + O_2^{\bullet -} \rightleftharpoons M^{n+} + OH^- + HO_2^-$$

$$H_2O_2 \rightleftharpoons H^+ + HO_2^-$$

$$M^{(n-1)+} + H_2O_2 \longrightarrow M^{n+} + OH^- + HO^\bullet$$

The last step is not an equilibrium reaction, as the hydroxyl radical formed, due to its extreme reactivity (*21*), will immediately react with the substrate.

According to this mechanism, the rate of formation of hydroxyl radicals, R, can be calculated from the expression:

$$R = q[M^{n+}][HO_2^-][H_2O_2]^{0.5}[O_2]^{-0.5}$$

where q is a collective constant. Using this expression, we would expect a maximum rate of hydroxyl formation in the pH range 11-11.5. Thus, current conditions of oxygen delignification seem to be nearly optimal for hydroxyl radical formation. It is interesting to note that, in principle at least, the rate of hydroxyl radical formation may be reduced by high oxygen pressure. The

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suggested mechanism for the formation of hydroxyl radicals may be regarded as a superoxide-driven Fenton reaction. The cyclic alteration of valence states is governed by the redox potential of the $M^{(n-1)+}/M^{n+}$ couple. Redox equilibria are generally proton-dependent. A transition metal may therefore act as a Fenton catalyst only within a certain pH-range. Under alkaline conditions, Mn and Cu catalyze the decomposition of hydrogen peroxide, whereas Fe is inactive. Conversely, Fe is an efficient catalyst in acidic solution, whereas Mn is inactive in this case (*22*).

The formation of hydroxyl radicals *via* one-electron reduction of hydrogen peroxide is catalyzed primarily by *mononuclear* transition metal ion species. In alkaline solution, such species may only arise when transition metal ions are present in very *low concentrations*. Accordingly, hydroxyl radical formation by reductive cleavage of hydrogen peroxide is most extensive with traces of transition metals. Under these conditions, Cu appears to be the most efficient Fenton catalyst (*23*). In *higher concentrations*, most metal ions aggregate or condense to form hydroxo-bridged polynuclear species in alkaline solution (*24*). For example, at pH > 9, Mn^{++} and HO^- ions form aggregates which can be oxidized by molecular oxygen to give MnO_2. Colloidal MnO_2 decomposes hydrogen peroxide efficiently by a two-electron reduction to give oxygen and water directly, *i.e.* without generating any significant amount of hydroxyl radicals. Colloidal particles of metal hydroxides and hydrated oxides may not only catalyze the decomposition of hydrogen peroxide, *i.e.* mimic catalase, but may also catalyze the dismutation of superoxide, *i.e.* mimic dismutase.

It has been repeatedly suggested that the hydroxyl radical, being a very strong oxidant [reduction potential = 2.32 V at pH 7 (*25*)], plays an important role in oxygen delignification. It reacts at diffusion-controlled rate by addition to aromatic nuclei (*21*) in phenolic as well as in non-phenolic structures and, in this way, contributes to lignin degradation. However, the hydroxyl radical also attacks carbohydrates by hydrogen atom abstraction at only marginally lower rate (*26*). For this reason, the hydroxyl radical is generally considered the main cause of carbohydrate degradation and loss of fiber strength properties. Due to its extreme reactivity, the hydroxyl radical is consumed at a site very close to its formation. Thus, hydroxyl radicals generated in the reaction liquor might only cause a small part of the viscosity drop (*27*). To put it in another way: It is not the hydroxyl radical *per se* that causes degradation of crystalline cellulose but the superoxide driven Fenton reaction running in the fibers that may result in a sustained attack leading to fiber fracture. Evidently, elimination of transition metals in the fibers is required to avoid extensive fiber degradation. Unfortunately, little is known about how transition metals are bound to the fibers.

The hydroxyl radical is also a weak acid with a pKa-value of 11.9 at 25°C (*21*). The anionic form, the oxyl radical, $O^{\bullet -}$, displays properties distinctly different from those of the hydroxyl radical. In contrast to the hydroxyl radical, the oxyl radical reacts predominantly by hydrogen atom abstraction. For this reason, the oxyl radical is probably less selective than the hydroxyl radical in its reactions with pulp.

Oxygen delignification became technically feasible when Roberts *et al.* (*27*) discovered that addition of magnesium compounds retards the degradation of cellulose more effectively than the degradation of lignin.

The protective effect of magnesium compounds can have different explanations, such as:

1. Coprecipitation of transition metal ions with magnesium hydroxide which should stabilize hydrogen peroxide against decomposition to give hydroxyl radicals (*28*) and achieve redox stabilization of Mn^{2+} (*22, 29*)
2. Formation of Mg-cellulose complexes which protect against attack by hydroxyl radicals (*30,31*).
3. Association of superoxide to the $Mg(OH)_2$ colloid may catalyze the proton dependent dismutation of superoxide , *i.e.* the $Mg(OH)_2$ colloid mimics superoxide dismutase:

$$[Mg_x(OH)_{2x}] + O_2^{\bullet -} \rightleftharpoons [Mg_x(OH)_{2x-1}\,O_2^{\bullet}] + OH^-$$
$$[Mg_x(OH)_{2x-1}\,O_2^{\bullet}] + O_2^{\bullet -} \longrightarrow [Mg_xO(OH)_{2x-2}] + HO_2^- + O_2$$
$$[Mg_xO(OH)_{2x-2}] + H_2O \longrightarrow [Mg_x(OH)_{2x}]$$

The first explanation has received wide acceptance but magnesium salts seem to act not only by deactivation of transition metal ions (*31, 32, 33*).

In oxygen and peroxide bleaching of pulp it is common practice to add chelators such as EDTA or DTPA to improve the viscosity. The effect of these chelators may be explained by the fact that metal ions in a higher valence state are more strongly complexed than ions in a lower valence state. Therefore, the reduction of chelated metal ions by superoxide to a lower valence state is inhibited, *i.e.* the superoxide-driven Fenton reaction is blocked.

The figure below summarizes how metal ion species may affect the four oxygen reduction steps. Only the superoxide driven Fenton reaction requires transition metal ion species. In other reduction steps metal ion species may have a catalytic effect.

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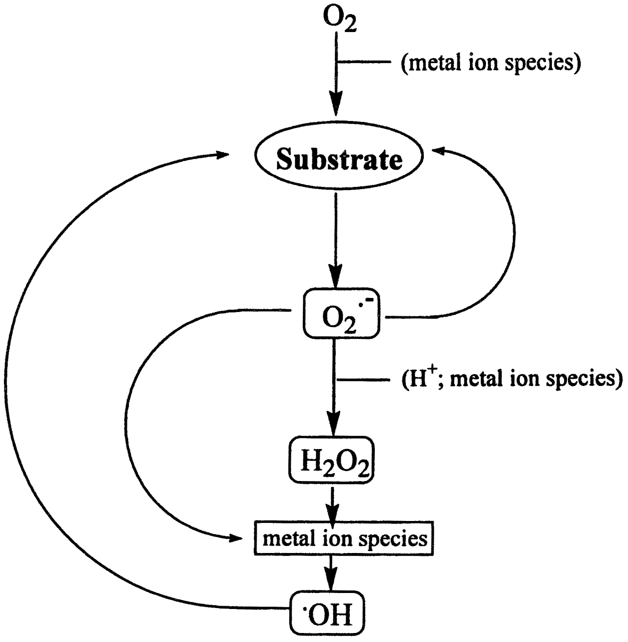

Figure 2. Catalysis by metal ion species in the oxygen reduction

Superoxide and Hydroxyl Radical Reactions with Lignin Model Compounds – γ-Radiolysis Studies

A significant part of our present understanding of the reactions of the hydroxyl radical and superoxide with lignins has been obtained in experiments where these species were generated in a controlled way by γ-radiolysis of aqueous solutions containing various types of lignin model compounds (*34-39*). A number of different reaction modes have been established by identifying major reaction products and by interpreting the pathways of their formation. The results of these studies can be summarized as follows:

1. *Hydroxyl radicals* readily oxidize phenolic structures to phenoxyl radicals. They also react with non-phenolic substrates by electrophilic addition to aromatic rings forming isomeric hydroxycyclohexadienyl adducts and by hydrogen abstraction from aliphatic residues affording carbon-centered radicals. In the presence of oxygen, these radical intermediates are oxidized

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to hydroxylation-, dealkoxylation- and C_{α}-C_{β} cleavage products, as well as to carbonyl-containing structures with concomitant formation of superoxide. Hydroxyl radicals alone can not degrade aromatic structures into aliphatic.

2. *Superoxide* readily reacts with phenoxyl and carbon-centered radicals. The reaction with phenoxyl radicals is characteristic of superoxide and is not given by molecular oxygen to any significant extent. This reaction may lead to ring opening or cleavage of a carbon-carbon bond in a ring-conjugated side chain (see Figure 3). Reaction products arising via ring opening, *e.g.* muconic acid and furane carboxylic acid derivatives conclusively show the formation of superoxide in oxygen degradation of lignin. In the absence of superoxide, phenoxyl (guaiacyl) radicals undergo radical coupling forming condensed phenolic units such as 5-5′biphenolic structures.

In oxygen delignification, reactions leading to ring opening or cleavage of side chains are of paramount importance since they cause lignin fragmentation and the introduction of carboxyl groups, which increase the solubility in alkali.

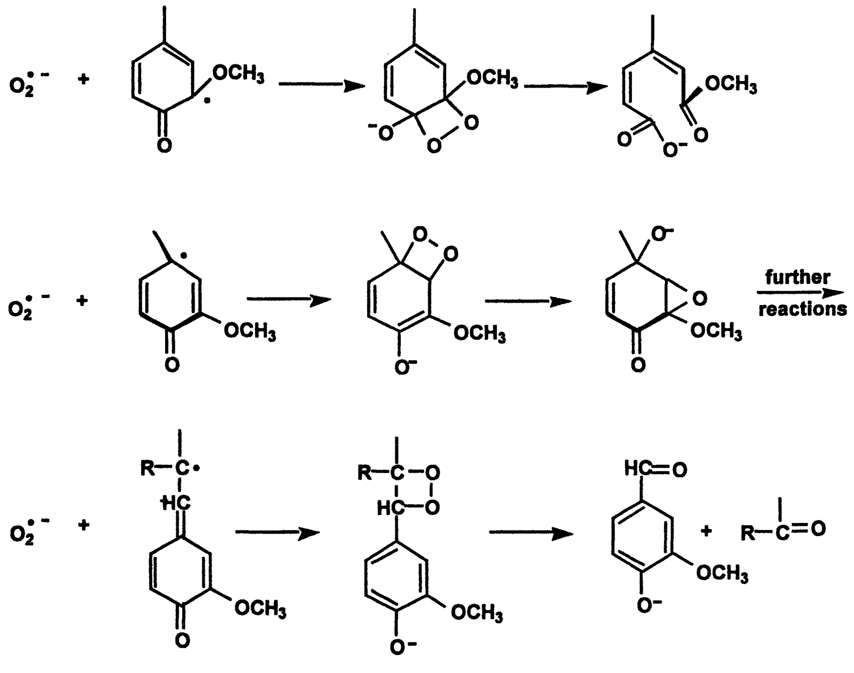

Figure 3. Superoxide dependant reactions.

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Formation and Involvement of Radicals in the Autoxidation of Lignin Model Compounds.

To validate the results of our radiolysis studies, we reacted the same lignin model compounds with superoxide and hydroxyl radicals generated during autoxidation [Gierer *et al.* in manuscript]. The effects exerted by different pH and by various metal ions on the formation of hydroxyl radicals and on the reactions of these radical species with model compounds were also studied. The figure below shows the product pattern *versus* pH after a 12 days autoxidation of *t*-butylsyringol (TBS) and *t*-butylguaiacol (TBG) at room temperature. As expected, the same major products were obtained by autoxidation as by treating these model compounds with radiolytically generated hydroxyl radicals and superoxide (*37*). In the following, only the formation of the major ring opening products, *i.e.* compounds of the muconic acid ester and furane acid types, will be discussed. During the work-up process, the muconic acid ester type of structure was readily transferred into the corresponding lactone type of structure by an acid-catalyzed cyclisation. The comparison of the yields is more reliable for each product at different pH values than between the different products, since calibration with authentic compounds was not always possible.

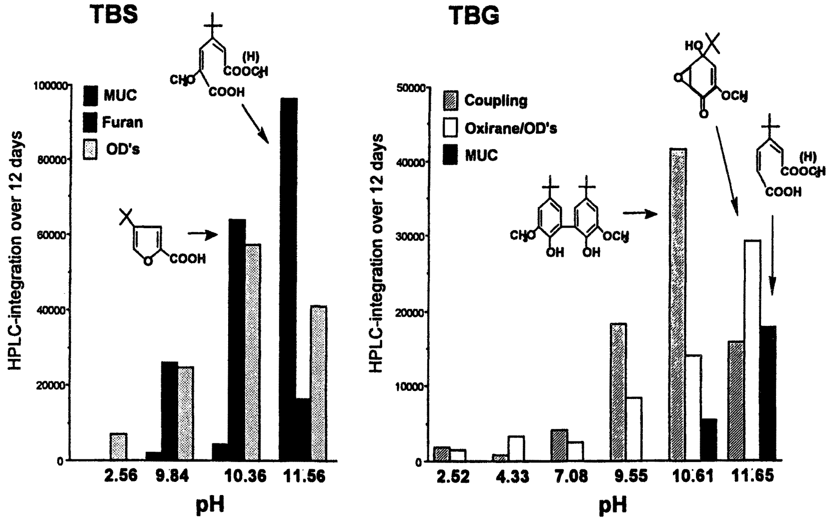

Figure 4. Autoxidation of TBS and TBG at room temperature. Product pattern vs. pH (OD's is the sum of other minor degradation products)

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In the case of TBS, the formation of ring opening products increases at the expense of the formation of the *t*-butylfurane carboxylic acid. These products may therefore arise *via* a common intermediate, an *o*-hydroperoxide (pKa-value about 11.6). Autoxidation at pH > 11.6 favors ring opening *via* the dioxetane mechanism, whereas autoxidation at pH< 11.6 leads predominantly to the formation of the furane carboxylic acid, presumably *via* hydrolytic cleavage of the hydroperoxide intermediate to give the *o*-quinone, and subsequent reaction steps (*40*). As expected, no coupling products were detected after autoxidation of TBS.

In the case of TBG, the dominant reaction product at pH < 11 is bis-*t*-butylguaiacol, whereas at higher pH the oxirane and the muconic acid derivative prevail. The latter two products arise *via* addition of superoxide to the mesomeric forms of the phenoxyl radical from TBG followed by ring closure and rearrangement of the resultant dioxetane. The coupling product bis-*t*-butylguaiacol is formed in competition with the addition of superoxide to the phenoxyl radical. The formation of the coupling product shows an interesting pH behavior indicating that there is another reaction consuming superoxide at pH<11. This may lend support to the suggestion that superoxide can oxidize phenols to the corresponding phenoxyl radicals by a concomitant proton and electron transfer reaction.

When subjected to the conditions of oxygen delignification (5 bar O_2, pH 10-13, 90 °C) non-phenolic lignin models remained unaffected. However, when a non-phenolic model was treated under the same conditions in the presence of a phenolic model significant degradation of the former was also observed. This demonstrates that during degradation of the phenolic model hydroxyl radicals are produced which attack the non-phenolic model. Formation of hydroxyl radicals was also confirmed with the chemiluminescence method (see below). Assuming that the hydroxyl radical attacks both models at equal (diffusion controlled) rate, the degradation of the non-phenolic model in relation to that of the phenolic model indicated a surprisingly efficient formation of hydroxyl radicals. This may be due to thermal instability of hydroperoxide intermediates:

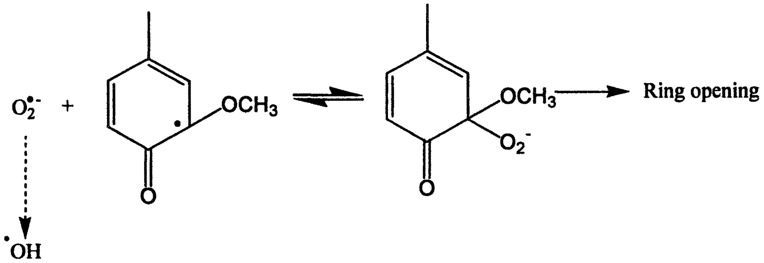

Figure 5. Proposed thermal instability of hydroperoxide intermediates leading to increased formation of hydroxyl radicals at 90° C

Accordingly, at 90 °C the superoxide driven Fenton reaction may prevail over reactions leading to cleavage of carbon-carbon bonds, *i.e.* ring opening or cleavage of side chains.

At all temperatures, the superoxide driven Fenton reaction was found the main source of hydroxyl radicals, *i.e.* homolytic cleavage of hydroperoxide is insignificant.

Hydroxyl Radical Formation in Pulp Bleaching and During Fungal Degradation of Wood.

To detect the formation of hydroxyl radicals in autoxidation reactions we have developed a very sensitive, yet selective, chemiluminescence method (*41, 42*). It is based on hydroxylation of non-chemiluminescent phthalic hydrazide by hydroxyl radicals to give the strongly chemiluminescent 3-hydroxyphthalic hydrazide

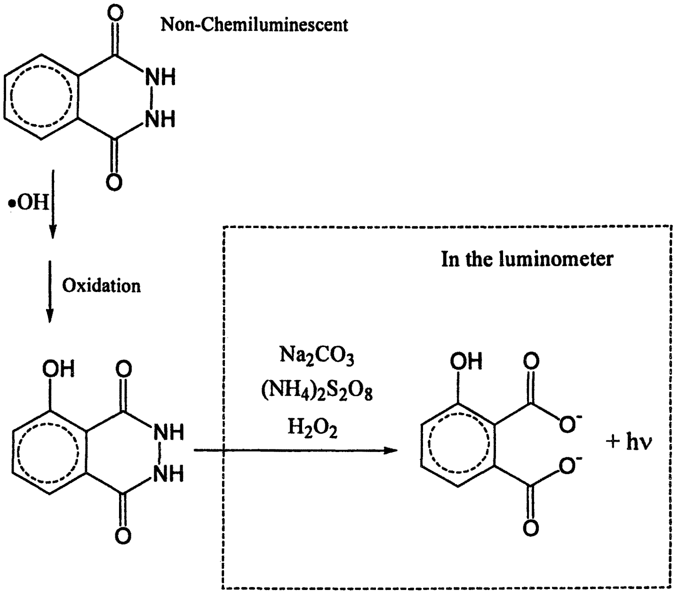

Figure 6. Chemiluminescence method to detect the formation of hydroxyl radicals.

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Due to the very high sensitivity of the method, only an insignificant amount of phthalic hydrazide is added to the reaction system.

Using this method we have been able to follow the formation and reactions of hydroxyl radicals during oxygen and hydrogen peroxide bleaching of pulp (*41*, *36*), as well as in methabolic processes associated with fungal growth (*43*, *44*). In wood shavings inoculated with brown or white rot fungi, a close correlation between hydroxyl radical formation and wood degradation was observed. These results led us to propose that hydroxyl radicals play an important part in the initial phase of microbial wood degradation, *e.g.* by cleavage of covalent bonds leading to partial fragmentation of the rigid structure. In addition, hydroxyl radicals may achieve other structural changes to facilitate the subsequent enzymatic breakdown of the fragments. However, only white-rot fungi produce oxidative enzymes capable of completing the breakdown of lignin.

Summary and Conclusions

Oxygen delignification is a free radical process governed by the interplay between superoxide and the hydroxyl radical. The reaction properties of these species complement each other:

$$\text{Superoxide} \quad \underset{O_2 + \text{substrate}}{\overset{H_2O_2 + M^{n+}}{\rightleftarrows}} \quad \text{Hydroxyl radical}$$

Mainly reducing	Strongly oxidizing
Selective	Unselective
Diffusible	Non-diffusible
Delignifying	Partly delignifying
Bleaching	Non-bleaching

One obvious limitation with oxygen delignification is the low reactivity of oxygen. The delignification proceeds at a reasonable rate only at high temperature and pH. However, these conditions favor carbohydrate degradation. Therefore, one would like to delignify with oxygen under milder conditions. This requires an alternative activation of the substrate using special oxidation catalysts, *e.g.* enzymes, transition metal complexes, polyoxymetalates or photocatalysts. In contrast to conventional oxygen delignification, these "advanced oxidation technologies" do not require high pH and temperature. For this reason, the cellulose fiber is expected to be less affected by deformation, "curl", and degradation by oxidation and alkaline hydrolysis.

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Chapter 16

Cell Wall Degradation of Spruce, Poplar, and Wheat Straw by MnO_2/Oxalate: An Overview

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Manganese dioxide and oxalate are two components commonly found in wood during its microbial decay. The degradation of cell wall from wheat straw, spruce and poplar sawdust by the association of MnO_2 and oxalate is reviewed here. The Mn oxidants formed are shown to modify the properties of the wood cell wall at the molecular but also ultrastructural levels. Marked reductions in the contents of ether linked Guaiacyl and Syringyl monomers, but also of the main dimers composing the lignins are observed in all samples after MnO_2/oxalate oxidation. The Mn oxidants also slightly modify polysaccharides, but in spruce only and probably at the level of hemicellulose. Finally, the cell wall architecture is altered by MnO_2/oxalate, in a specific manner depending on the plant considered.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Introduction

Wood-destroying fungi accumulate large amounts of transition metals during decay *(1 ,2)* and black MnO_2 deposits are frequently observed on fibers in severely delignified wood *(3, 4)*. They also produce significant levels of oxalic acid during the process, which can sometimes acidify wood down to pH 2 *(5, 6)*. However, the role of manganese(IV) as MnO_2 species in microbial degradation and the reasons for oxalate accumulation in woods remain hypothetical *(2, 7, 8)*.

We have recently shown that the solubilization of MnO_2 by oxalate at pH=2,5 leads to the production of Mn chelates capable of oxidizing lignin within wheat straw, poplar and spruce cell wall *(9, 10, 11 ,12)*. We therefore proposed that MnO_2 and related Mn(IV) species could indeed be actively involved in the degradation of lignocellulose when associated with oxalates, in complement to the well described enzymatic processes. Still, such a mechanism and the possible cooperations between abiotic and enzyme systems are not demonstrated to occur in the natural environment, but all elements are in place within wood to promote this phenomenon.

This paper reviews the results on the ability of the Mn/oxalate oxidative system to selectively modify lignocellulosic matrixes. Specific degradation pattern of lignin, cellulose and hemicelluloses were evidenced in wheat straw, poplar and spruce wood under the same experimental conditions, and ultrastructural modifications pointed out the importance of the supramolecular organization of cell wall on its degradation.

Experimental

Plant material and substrates

Wheat straw (*Triticum aestivum sp.*) was harvested by hand at full maturity and air dried. Internodes were separated, collected and reduced into ~2mm particle size. Extractive free poplar wood sawdust (*Populus trichocarpa,* cv Fridzi Pauley) and spruce wood (*Picea abies*) sawdust was used throughout the study. Permethylation of *in situ* lignin in wheat straw was carried out by diazomethane generated from Diazald® (Sigma Aldrich) *(13)*. Before oxidation a short four-minute ball milling was applied on the sample in order to reduce heterogeneity between particle size *(10)*.

Chemicals

Activated MnO_2 (85% ; particle size <5 μm) Na-oxalate and oxalic acid were purchased from Sigma Aldrich (France). Other reagents and solvents used were of analytical grade.

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Oxidative treatment with MnO_2

The straw, poplar or spruce (21.0 ± 1.3% ; 22 ± 4% ; 27.8 ± 0.2% Klason lignin content, respectively) were incubated at a concentration of 5mg/ml for poplar or spruce, and 10 mg/ml for wheat straw for 20 hours at room temperature (20 to 25°C) under stirring in Na-oxalate buffer containing MnO_2. Different reaction conditions were studied over the years and the followings will be discussed in this paper.

- **A**: 1 equivalent (equiv.) lignin on the basis of MW= 230 ; 20 equiv. MnO_2; 40 equiv. oxalate buffer pH 2.5
- **B**: 1 equiv. lignin; 10 equiv. MnO_2; 20 equiv. oxalate buffer pH 2.5
- **C**: 1 equiv. lignin ; 5 equiv. MnO_2; 10 equiv. oxalate buffer pH 2.5
- **D**: 1 equiv lignin; 1 equiv. MnO_2; 2 equiv. oxalate buffer pH 2.5

Two control experiments were performed. The first one consisted in the incubation of samples in 100 mM oxalate buffer pH 2.5; the second one, in samples incubated in water.

At the end of the reaction period, the samples were recovered by vacuum filtration and washed with oxalate buffer (100 mM pH 2.5) to remove black MnO_2 deposits, and with hot water to remove white precipitates which were formed during the reaction. The samples were freeze-dried before chemical analysis. For microscopic analysis, dehydration was performed as previously described *(14)*.

Chemical analysis

Lignin content and characterization

The Klason lignin content in samples was estimated according to a procedure similar to that described by Effland *(15)*. The lignin fraction is accounted as the acid insoluble fraction depleted from its ashes.

The content of β-O-4 linked monomers and dimeric structures was determined by thioacidolysis on the lyophilized sample. The monomers and dimers released (**figure 1**) were separated by capillary gas chromatography as trimethylsilyl derivatives and identified by gas chromatography/mass spectrometry (GC/MS ; electronic impact, ion-trap instrument) *(16)*. The total yields of the main Guaiacyl (G) and Syringyl (S) monomers reflect the amount of such units only involved in β-O-4 bonds. The main G-G, S-S and G-S dimers recovered after thioacidolysis and then Raney nickel desulfurization are representative of the various carbon-carbon and diarylether bonds in the polymer, referred to as the " condensed " bonds (**figure 1**).

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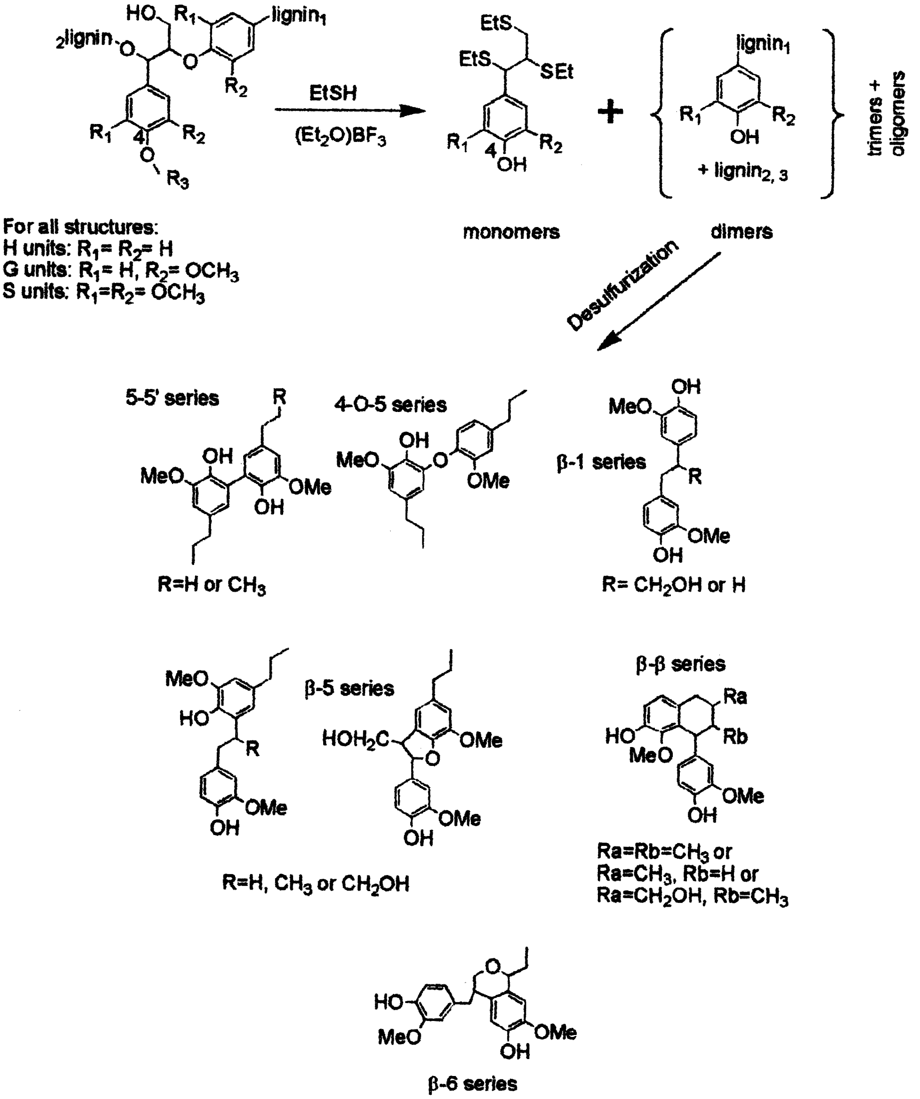
HO
R1
lignin1
2lignin
O
O
R2
R1
4
R2
O
R3
EtSH
(Et2O)BF3
EtS
EtS
SEt
R1
4
OH
R2
+
lignin1
R1
R2
OH
+ lignin2, 3
trimers + oligomers
For all structures:
H units: R1= R2= H
G units: R1= H, R2= OCH3
S units: R1=R2= OCH3
monomers
dimers
Desulfurization
5-5' series
R
OH
MeO
OMe
OH
R=H or CH3
4-O-5 series
OH
MeO
O
OMe
OH
MeO
β-1 series
R
OMe
OH
R= CH2OH or H
MeO
HO
R
OMe
OH
β-5 series
HOH2C
OMe
O
OMe
OH
R=H, CH3 or CH2OH
β-β series
Ra
HO
Rb
MeO
OMe
OH
Ra=Rb=CH3 or
Ra=CH3, Rb=H or
Ra=CH2OH, Rb=CH3
O
HO
MeO
OMe
OH
β-6 series

Figure 1 : Depolymerization of lignin by thioacidolysis for the analysis of β-O-4 linked monomers and dimers.

Polysaccharide composition

Polysaccharides in samples were hydrolyzed by H_2SO_4 according to *(17)*. Fucose was added as an internal standard just after the pre-hydrolysis step. The monosaccharides released by the treatments were analyzed by high performance anion-exchange chromatography with pulsed amperometry detection, as described previously *(9)*.

Phenolic acid analysis

The content in ester and ether linked p-coumaric and ferulic acids was determined on wheat straw sample after successive alkaline hydrolysis and acidolysis, respectively according to Scalbert *(18)*.

Microscopy

Observation at the macroscopic level was done by optical microscopy without staining of the freeze dried sample. Scanning electron microscopy (SEM) at medium magnification level was used to analyze the surface aspects of the freeze dried samples. Transmission electron microscopy (TEM) was performed on ultrathin sections of dehydrated samples embedded in resin. The tissues were stained for polysaccharides with the periodic acid-silver reagent of Thiery adapted by Ruel *(19)* or with potassium permanganate *(14)*.

Statistical analysis

The experiments were performed independently at least four times. Each set of experiment comprises two control samples incubated in water, two samples incubated in oxalate and two samples incubated in oxalate plus MnO_2. Statistical analysis were performed by comparing data obtained from two populations, defined as control samples (incubated in water, or in oxalate) and as treated sample (treated by oxalate alone, or by oxalate plus MnO_2). The probability p for the two populations to be identical was determined using an unilateral Student test. The treated samples were considered to be different from the control sample for $p < 0.05$.

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Results and discussion

Modification of cell wall structure by MnO_2/oxalate

Microscopic analysis of wood sawdust and of wheat straw was difficult due to the heterogeneity in size and structure of the particles. Therefore, only fragments with apparent structures were considered. Different levels of modification could be evidenced (**figures 2 to 4**).

At a macroscopic scale, only poplar showed strong alteration of the regular pattern of parenchyma ray cells oriented perpendicularly to fiber structures (arrows ; **figure 2 A and B** ; conditions A).

The main effect of MnO_2/oxalate in conditions C on spruce was a removal of some amorphous structures from the surface of the lumen-side cell wall of tracheids, where a characteristic radial fibrillar organization then appeared (arrows f, **figure 3 A and B**). Some needle shaped oxalate crystals were also found, but only in oxidized samples treated with MnO_2 despite the extensive washing performed.

In wheat straw, modifications were only evidenced at the ultrastructural level. After oxidation by MnO_2/oxalate (conditions C), PATAg staining for polysaccharides showed two types of modifications (**figure 4 A and B**). Highly contrasted regions in controls became less densely contrasted in oxidized samples (arrows a) and layers less reactive in controls became clearer and exhibited a fibrillar organization (arrows b). Staining with $KMnO_4$ also revealed a slight disorganization of the lignin network within the secondary cell wall (**figure 4 C and D**). For instance the S1 and S2 layers show a carded appearance not found in the sample incubated in water or treated by oxalate. In general, cell corner reactivity did not change after oxidation.

Chemical modifications within cell wall catalyzed by MnO_2/oxalate

Despite visual changes in the oxidized wood or wheat straw by microscopy, no lignin and polysaccharide removal could be quantified in wheat straw and poplar samples, indicating that MnO_2/oxalate is not a delignifying or an hydrolytic system (**Table I**). In all cases, no degradation was observed when oxalate buffer alone was used (data not shown; Gaudard and Kurek, unpublished; ref (10), (11), (12)).

The only exception is spruce, where a slight but significant 8% loss of arabinose and galactose and a 10% loss of glucose occurred after MnO_2/oxalate oxidation (**Table I**). Thus, hemicelluloses and possibly cellulose were slightly

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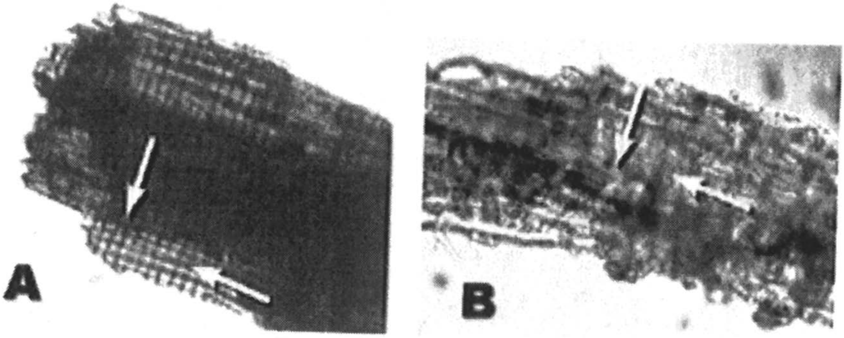

Figure 2. Optical microscopy of poplar wood sawdust oxidized by MnO$_2$/oxalate. Magnification= 40x ; (A) : control incubated in water ; (B) : oxidized sample in conditions A

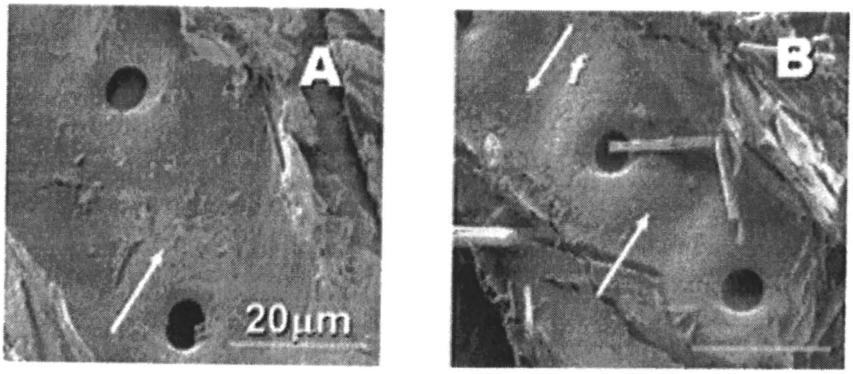

Figure 3. SEM of spruce wood tracheids in sawdust incubated in water (A) and after oxidation by MnO$_2$/oxalate (B) in conditions C. (A) : the lumen-side wall of tracheid appears as a rough surface bearing amorphous structures (arrow); (B) the oxidized tracheids present a clean and smooth surface; some underlying radial (arrow) fibrillar organization is also observed.

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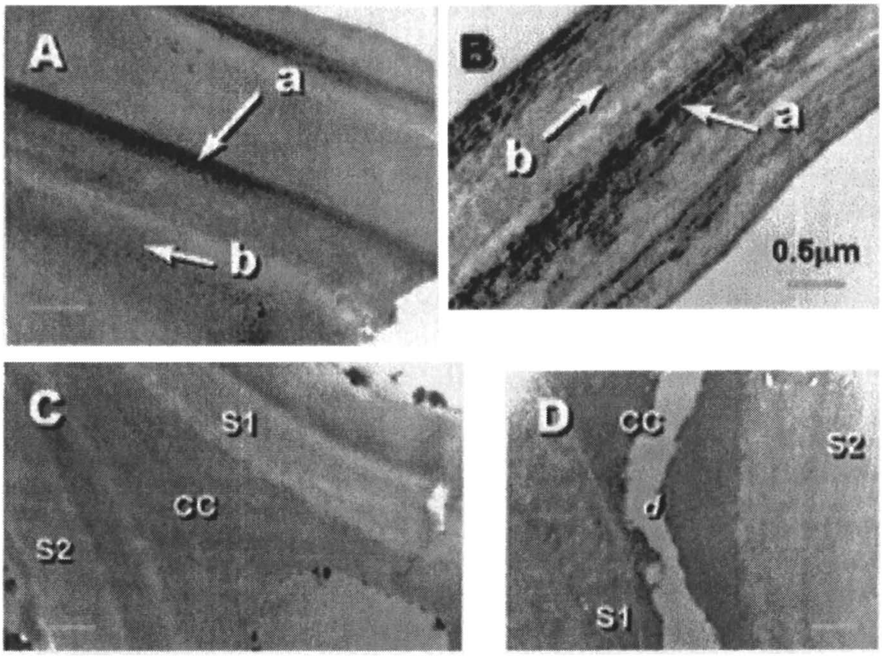

Figure 4. TEM of wheat straw incubated in MnO_2/oxalate in conditions C. (A) and (B) : main features of fiber cell wall fragments stained with the PATAg method. (A): control wheat straw incubated in water ; note the sharp edges without visible degradation in fiber; note the concentric heterogeneity of the distribution of the polysaccharides with strongly labelled layers (arrow a). (B): vessel wall and adjacent fiber treated by MnO_2/oxalate; general clearing of the different layers (arrows a and b) with fibrillated organization compared to the sample incubated in water. (C) and (D) : modification by MnO_2/oxalate revealed by $KMnO_4$ staining of two adjacent fibers mechanically disrupted (d) at the level of the middle lamela and cell corner (CC). The staining of the cell corner (CC) in the oxidized sample (D) is unmodified, compared to control (C); the S1 and S2 layers exhibit a characteristic carded aspect in oxidized samples not found in control.

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Table I. Poplar and spruce sawdust composition before and after oxidation by MnO_2/oxalate

	Poplar		Spruce	
(mg/g)	Control[a]	Oxidized [b]	Control[a]	Oxidized [c]
Lignin	223.8 ± 4	221.7 ± 4 (NS)[d]	278 ± 2.1	279 ± 8.3 (NS)
Glucose	471.2 ± 23	465.3 ± 23 (NS)	444 ± 14.9	398 ± 31 (S)[e]
Mannose	19.1 ± 0.8	18.4 ± 1.3 (NS)	113 ± 17.8	105 ± 17.1 (NS)
Xylose	145.4 ± 7	142.9 ± 7 (NS)	46 ± 11	45 ± 13 (NS)
Galactose	42 ± 0.6	49 ± 0.7 (NS)	14 ± 0.9	13 ± 1.1 (S)
Arabinose	23 ± 0.3	26 ± 1 (NS)	9.8 ± 0.6	8.8 ±0.5 (S)

[a] sample incubated in water ; [b] conditions **B**, from ref (10) and from Gaudard and Kurek, unpublished, [c] conditions **C**, from ref (12), [d] NS: difference between oxidized and control samples not significant at $p < 0.05$; [e] S: differences significant at $p < 0.05$.

modified in this case. This specific degradation pattern may reflect the anatomical differences with poplar and wheat straw, as well as the difference in the composition of hemicelluloses *(20)*.

The major impact of MnO_2/oxalate treatment on woods and wheat straw was found to be on aromatic structure: the lignins and phenolic acids.

Indeed, the bonding patterns between the constitutive Guaiacyl and Syringyl units were severely altered during oxidation. A marked reduction in the monomers as well as in the various dimers recovered after the selective chemical cleavages of β-O-4 bonds by thioacidolysis was observed (**figure 5**, **Table II**), showing that all these structures were oxidized by the Mn complexes.

Degradation of lignins may however proceed through oxidation of phenols. Indeed, the MnO_2/oxalate system was inactive on lignin which has been extensively permethylated *in situ* (data not shown, *(9)*). The presence of free phenols on lignin monomers is thus required for efficient MnO_2/oxalate catalysis. This also confirms that lignin attack may proceed through oxidation of the aromatic moiety, as described for Mn(III)-chelates generated enzymatically by Mn peroxidase, but probably not through side chain oxidation, the common pathway for solid MnO_2 *(21)*.

Table II. Extent of degradation of dimers in poplar, spruce and wheat straw by MnO_2/oxalate

	dimers[a]					
	β-1	β-5	β-β	β-6	4-O-5	5-5
poplar + MnO_2 /oxalate [b]	78	80	54	nd	58	51
spruce + MnO_2 /oxalate [c]	40	ns	18	45	ns	22
wheat straw + MnO_2 /oxalate [d]	25	17	12	nd	++[e]	0

[a] % degradation relative to control sample incubated in water; deviations to mean < 10%; [b] conditions **B**, from *(10)* ; [c] conditions **C**, from *(12)* ; [d] conditions **C**, from *(11)* ; [e] a significant 18% higher recovery yield was obtained, relative to control ; nd : not determined.

On the other hand, taking into account the proportion of phenolic S units in the original wheat straw and poplar lignin, (~5% ; *(22,13)*), higher degradation yields of S units (15 to 20%) indicated that phenolic as well as non-phenolic β-O-4 linked S monomers were oxidized by MnO_2/oxalate. Thus, it is likely that the degradation within non-phenolic domains proceed through the formation of new

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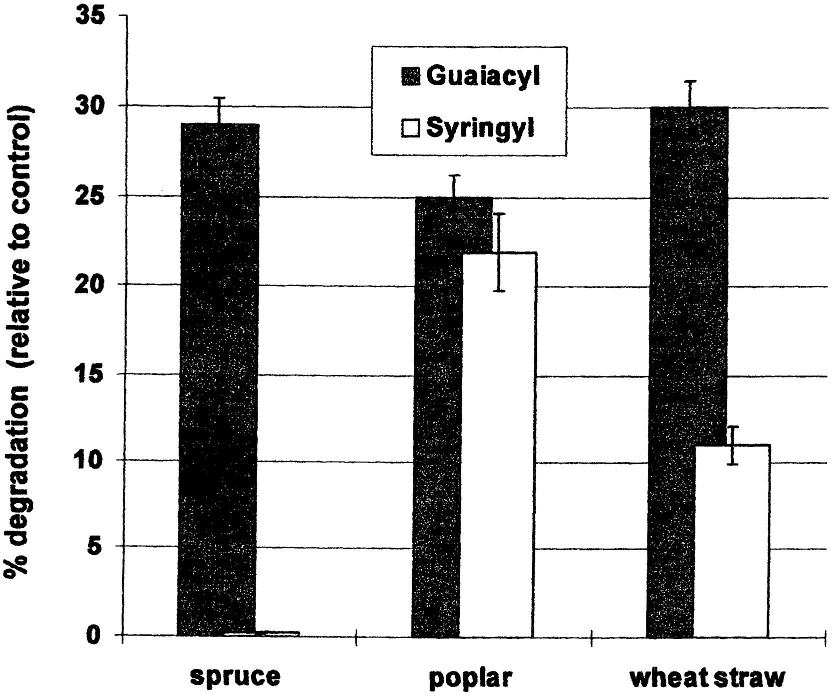

Figure 5. Degradation of ether linked monomers of lignins in samples oxidized by MnO_2 / oxalate in conditions C

phenolic sites by breaking off some of the β-O-4 bonds adjacent to the phenolic moieties attacked by MnO_2/oxalate *(23)*.

The dimeric structures in lignins were also degraded, but at different extent, according to the type of intermonomer bonds (**Table II**). As previously observed with lignin oxidizing enzymes acting on spruce lignin *(24)*, the β-1 structures are more easily oxidized than the others in all plants tested. On the other hand, the main β-5 and the minor 4-O-5 structures were not, or only to a low extent, degraded by MnO_2/oxalate in spruce and wheat straw, as opposed to poplar (see **Table II**). These differences may again be due to topochemical factors related to the supramolecular organization of the cell wall matrix.

A particular aspect of graminaceous cell walls is the presence of cinnamic acids, with monomers linked to lignin and/or polysaccharides *(25)*. Different reactivity of such cinnamic acids towards abiotic lignin oxidizing agents was evident considering their disappearance rate in oxidized wheat straw (**Table III**). Indeed, ester linked phenolic acids were more reactive than ether linked ones and ferulates were always prone to be oxidized, compared to coumarates. In particular esterified ferulate was almost completely depleted from the sample oxidized with 50 mM MnO_2 in the presence of 100 mM oxalate (conditions **C** ; **Table III**).

Table III: Wheat straw content in ferulic and *p*-coumaric acids

	Ferulic acid [a]		*p*-Coumaric acid [a]	
	esterified	etherified	Esterified	Etherified
control sample[b]	14.1±0.6	14.6±1.9	21.9±0.5	4.0±0.5
Oxidized - condition **C**[c]	0.42	10.9	13.6	3.2
Oxidized - condition **D**[c]	4.5	12.0	20.1	3.9

[a] in μmoles/g sample ; [b] incubated in oxalate ; [c] data from ref *(9)*; deviations to mean < 15%.

However, no free phenolic acids have been detected in the reaction media (data not shown, *(9)*). It is likely that oxidized cinnamates may be retained within the cell wall, probably through overreaction with lignin, forming new structures which escape to classical chemical analyses. In this respect, ferulic acid was shown to be readily polymerized by MnO_2/oxalate in condition **B** (*(9)*; data not shown).

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Conclusions

The different studies presented here demonstrated the degradative action of the abiotic oxidative system composed of MnO_2 and oxalate on wood and graminacea.

The modifications at the level of cell wall ultrastructure, the strong modifications in the lignin bonding pattern in each plant considered, the specific alteration of cinnamic acids in wheat straw and the slight but significant degradation of hemicelluloses in spruce wood confirm that abiotic Mn chelates are able to modify the supramolecular organisation of the cell wall matrixes, and some of the particular interactions existing between the constitutive polymers.

Thus, MnO_2 and oxalate could be part of an efficient system that pretreats lignocellulosic material before or during microbial and/or enzymatic attack of cell walls. However, further studies are required in order to demonstrate the existence of such a mechanism *in situ* and *in vivo*, possibly controlled by or coordinated with the action of ligninolytic microorganisms.

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Chapter 17

Metal–Schiff Base Complexes: Useful Mimics for Phenol Oxidants in Catalytic Delignification?

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The catalytic effect of metal-Schiff base complexes in phenol oxidation reactions has been investigated. In the first experimental set the effects of the metal and the structure of the ligand on the catalytic oxidation reactions were studied using salen type complexes and following the oxidation of coniferyl and sinapyl alcohols. The most promising complexes were then examined with monomeric and dimeric lignin models in systems relevant to practical bleaching conditions. The results indicate that Schiff-bases catalyze the oxidation of lignin model compounds either by one- or two-electron oxidation mechanism and that the mechanism is dependent on the bulk of the substrate. Sterically bulky β-ether models reacted by formation of biphenyls whereas monomeric models with benzyl alcohol structures gave products with benzylic carbonyl groups. Only small amounts of quinoid structures were found among the reaction products.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Introduction

In recent years increasing environmental concerns have forced industry to develop "green" technologies in their production processes. In the pulp and paper industry most concerns have been focused on bleaching where alternative bleaching agents are required to replace chlorine containing reagents (*1*). Today several techniques in this field are already a reality. Oxygen, ozone and hydrogen peroxide used in sequences, provide commercially available bleaching technologies (*2*) and xylanases have been used successfully to enhance lignin removal from pulps (*3*). The use of oxidation catalysts such as laccase with mediators (*4*), polyoxometallates (*5*) and binuclear Mn-complexes (*6*) have shown to be potentially available technologies for chlorine free- bleaching.

Laccase, polyoxometallate and binuclear Mn-complex approaches in bleaching are based on the idea of using "biomimetic" procedures for residual lignin degradation. However, although the published material relating to these procedures indisputably show their positive effects on bleaching parameters, little is known so far on their detailed action in lignin removal from pulp. In the present study we have focused particularly on the chemical aspects involved in "biomimetic" approaches in bleaching. For this purpose we have mimicked the oxidation capacity of different enzymes known to be relevant in lignin biodegradation (*7*) by synthetically obtained oxidation catalysts, so called metal-salen compounds of type **1** and **2**. The salen compounds are structurally simple and easy to handle allowing the use of conventional practical organic chemistry methods for the studies.

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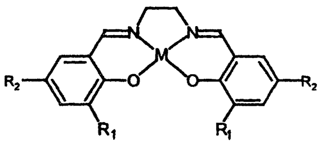

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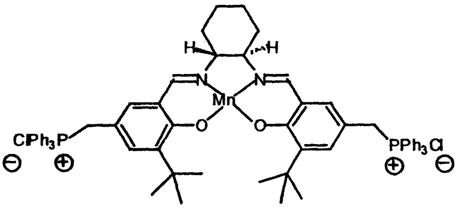
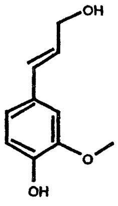


2

a) $R_1 = R_2 = H$
b) $R_1 = H$, $R_2 = SO_3Na$
c) $R_1 = t\text{-Bu}$, $R_2 = CH_2PPh_3Cl$

M = Mn(III), Fe (III), Co(II), Cu(II)



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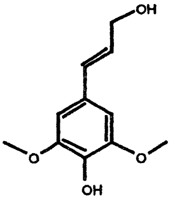

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Results and Discussion

Dehydrogenative polymerization of coniferyl and sinapyl alcohols catalyzed by metal-salen complexes

The salen complexes **1** and **2** were prepared according to literature procedures (*8*) with slight modifications and the metal center was introduced using standard methods (*9*).

In a typical oxidation experiment **3** (coniferyl alcohol) or **4** (sinapyl alcohol) was dissolved in 2:1 water-dioxane solution buffered to pH 3. Low pH was used to mimic the activity maximum of lignin peroxidase (*10*). The salen complex (Co, Mn or Fe, 0.05 mol. equiv.) and H_2O_2 (1 mol. equiv., in three portions) were then added to this solution and the reaction followed by TLC until the disappearance of the substrate. In experiments where the oxidant was oxygen (Co and Cu), the reaction solution was stirred either in an open flask or with slight overpressure of dioxygen using a balloon. In some instances imidazole was used to mimic the axial ligand of enzymes (*10*). The DHP (synthetic lignin) formed in the oxidation was separated from the reaction solution by extraction with ethyl acetate, acetylated and analyzed by NMR. The DHPs were compared to those produced by HRP (horseradish peroxidase)/H_2O_2 oxidation system. The results obtained in experiments with **3** are presented in Table **1**.

The results indicate that, in general, metal salen compounds show substantial phenol oxidative capacity under conditions assumed to prevail during lignin bioconversions. The order of reactivity seems to be roughly Fe ≈ Co > Mn >> Cu. With copper, coniferyl alcohol was oxidized only in the case of bulky **1c**. The long reaction time with Cu**1c** is in accordance with the findings that copper containing enzymes, like laccase, has been reported to take several days and to stop at the dimeric stage (*11*).

The effect of imidazole, a mimic for internal ligands in enzymes, is also pronounced: when added to the reaction mixture it significantly accelerates the

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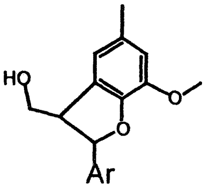
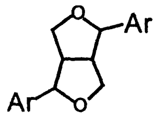
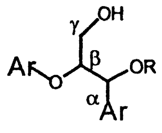


β-5



β-β



β-O-4

Table 1. Dehydrogenative polymerization of coniferyl alcohol (CA) in aqueous dioxane at pH 3 (oxidant H_2O_2)

Catalysts	β–5:β–β:β–0–4	Time for CA disappearance
Mn1a	2:3:2	18 h
Mn1b	2:3:1	10 h
Mn1c	3:2:1	35 min
Mn2	2:2:1	1 h
Fe1c	2:2:1	2 h
Co1a	3:3:1	35 min
Cu1c	1:1:1	3 days
Mn1a + imid.	3:3:1	1.5 h
Fe1a + imid.	3:3:1	15 min
Co1a + imid.		no reaction
Cu1a + imid.		no reaction
HRP	2:2:3	1 h

oxidation. In general, oxidations with salen catalysts seem to take place faster than with HRP. The results further indicate that increasing the bulk in the ligand results in faster oxidations. One explanation might be that bulky substituents increase the catalytic effect by preventing the ligand from settling into a planar conformation. In the case of **Cu1c** we have been able to show the twisted conformation by X-ray analysis (Figure **1**) (*12*).

An increase in the bulk of the ligand in the case of Mn has only a slight effect on the product distribution. This was also the case with other metals. In the case of **Co1a**, changing the oxidant from hydrogen peroxide to oxygen did not change the product distribution.

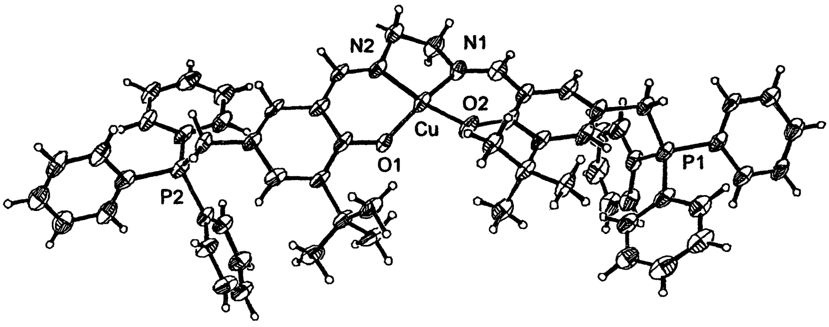


Figure 1.

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The β-5 dimer formed upon oxidation of **3** with the chiral complex **2** was investigated by chiral HPLC and found to be racemic. The lack of regiospecifity and enantioselectivity in the coupling reactions indicate a random radical coupling mechanism in salen catalyzed dehydrogenation reactions in aqueous solutions.

In experiments with sinapyl alcohol we were particularly interested in finding an explanation why sinapyl alcohol produces β-O-4 -rich DHP when oxidized by $FeCl_3$ but gives predominantly β-β dimers in hydrogen peroxide/HRP systems (*13*). One explanation for this phenomenon might be that in $FeCl_3$ oxidation the HCl liberated catalyzes the addition of water to β-O-4-type quinone methides and forces the system to produce β-O-4 type polymer (*13*). Indeed, at lower pH the polymerization of **4** using **Mn1a**/hydrogen peroxide oxidation system gave larger amounts of β-O-4 type structures than the experiments with HRP/hydrogen peroxide system at pH 3.

Oxidation of monomeric lignin model compounds catalyzed by Mn- and Co-complexes

The experiments described above indicate that Fe, Mn and Co all form salen-type complexes capable of catalysing the oxidation of phenolic materials. We decided to concentrate on Mn and Co as some results have suggested that hydrogen peroxide oxidations with Fe cause unwanted side reactions, such as production of hydroxyl radicals.

To examine our catalysts under bleaching conditions, we first studied the activity of Co-catalysts in the oxidation of benzyl alcohols **5** (vanillyl alcohol) and **7** (veratryl alcohol) with dioxygen in aqueous solution at pH 10 at two temperatures, 80 and 100 °C. In addition to Co-salen **Co1a** and Co-sulphosalen **Co1b** we also examined the reactivities of Co-acacen **9**, Co-α-methylsalen **10**, Co-4-hydroxysalen **11**. Conversions to corresponding

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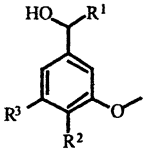
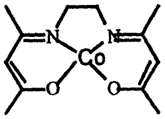


5 $R^1 = H, R^2 = OH, R^3 = H$
6 $R^1 = Me, R^2 = OH, R^3 = H$
7 $R^1 = H, R^2 = OMe, R^3 = H$
8 $R^1 = Me, R^2 = OMe, R^3 = H$



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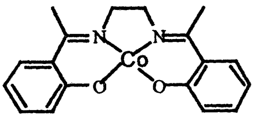
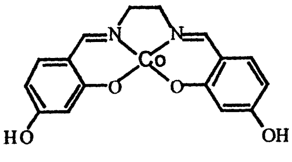


10


N
N
Co
O
O
HO
OH

11

aldehydes (the only products found apart from a small amount of polymeric material) were followed by ^{1}H-NMR (Table 2). The results were then compared to ambient temperature oxidations using Mn**1b** with hydrogen peroxide and Co**1a** with dioxygen. These were performed in 1:1 methanol-water solvent systems, at pH 10 (Table 3).

Table 2. The oxidation of (5) and (7) oxidised by dioxygen and catalysed by Co-complexes at pH 10

Catalyst	Substrate	80°C (% Conv.)	100°C (% Conv.)
1a	**5**	83	91
1b	**5**	38	86
9	**5**	27	95
10	**5**	6	88
11	**5**	53	60
1a	**7**	25	71
1b	**7**	22	43
9	**7**	21	28
10	**7**	28	25
11	**7**	4	71

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The unsubstituted Co-salen **1a** seems to work best, giving the highest conversions and practically showing no temperature effect between 80 °C and 100 °C with the substrate **5** (Table 2). The result is somewhat surprising as one might expect catalyst **1b**, with electron attracting sulphonyl groups, to exhibit enhanced catalytic properties compared to **1a** (and also to **11**) (*14*). The results also show that the change in temperature has a significant effect only in the case of vanillyl alcohol **5**; the conversion of non-phenolic veratryl alcohol **7** to aldehyde remained practically at the same level at both temperatures (80 °C and 100 °C). Most probably this phenomenon is due to the presence/absence of a free phenolic group in the substrate.

In the experiments with Mn-hydrogen peroxide system (Table 3), the conversions of structurally most simple benzyl alcohols **5** and **7** are about the same level as in oxidations with Co-catalysts at 80 °C (Table 2). For some reason, compound **6** was completely unreactive under the reaction conditions. Also, the Co-catalyst Co**1a** seems to lose most of its activity at the lower temperature.

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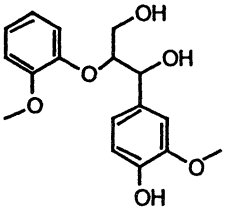
Table 3. Oxidation of "monomeric" models 5 – 8 by Mn-sulfosalen 1b and Co-salen 1a at ambient temperature at pH 10.

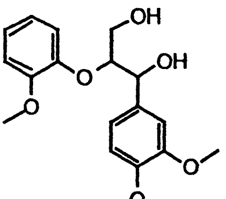
Catalyst	Substrate	Conversion (%)
Mn1b	**5**	70
Mn1b	**6**	0
Mn1b	**7**	65
Mn1b	**8**	50
Co1a	**5**	10

Oxidation of dimeric lignin model compounds catalyzed by Mn- and Co-complexes

The experiments with dimeric lignin models were performed in 1:1 water-methanol solution (pH 10). The molar ratio of substrate to catalysts was 1:0.05. In studies with **Mn1b** the oxidant was hydrogen peroxide and the reactions were performed at room temperature. For **Co1a** and **Co1b**, the oxidant was dioxygen and the reactions were performed under refluxing conditions, with a slight overpressure of dioxygen. The products were identified by NMR.



12



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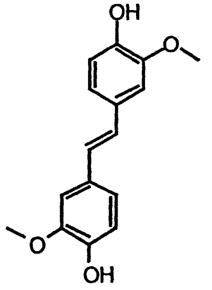
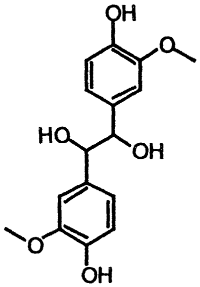
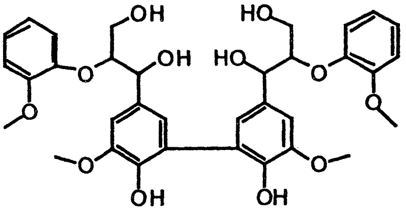


14

Oxidation of dimer **12** with **Mn1a** yielded biphenyl **16**, a radical coupling product, as the sole product. The oxidation of **12** with **Co1b** gave back starting material. With **Co1a**, ^{1}H NMR showed weak signals around 11 ppm and 9 ppm suggesting minor degradation of the substrate. Dimer **13** turned out to be totally unreactive in the reaction systems investigated. Even the addition of pyridine as an axial ligand (*15*) in experiments with Co**1a** and **1b** had no effect suggesting the total inertness of non-phenolic dimers in the oxidation system.



15



16

To elucidate the reactivity of chromophoric structures in residual lignins we then studied the reactivity of (*E*)-4,4'-dihydroxy-3,3'-dimethoxystilbene (**14**). The manganese complex **1a** quantitatively oxidised **14** to diol **15**. When the oxidation was performed with Co**1a** or Co**1b**, a considerable amount of vanillin was formed as indicated by a singlet at 9.78 ppm in the ^{1}H NMR spectrum of the oxidation mixture.

Conclusions

The results of this study demonstrate that both Mn- and Co-salen catalysts have clear capacity to catalyse phenol oxidation under conditions applicable to bleaching processes. The results indicate that in aqueous solvent Mn catalysts with hydrogen peroxide are able to oxidize lignin models either to form carbonyl compounds or radical coupling products. With structurally simple lignin models Mn-salens catalyze the oxidation of the benzylic position to a carbonyl (a 2-electron oxidation) whereas in the case of bulky substrates, one-electron oxidation predominates and biphenyl structures are formed. Cobalt catalysts, on the other hand, seem to oxidize model compounds to give carbonyl structures. The Co-catalyzed oxidations, however, seem to be restricted to monomeric substrates. The recent findings in the field of bleaching chemistry has established that residual lignins contain substantial amounts of β-ether

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structures, and that catalysts such as the salen compounds clearly assist removal of residual lignin from the pulps. Our results, however, demonstrate that these catalysts will preferentially polymerize bulky substrates like β-ethers in alkaline solutions. Further studies are therefore required to reveal the relevant mechanisms of catalytic bleaching.

Acknowledgement

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Chapter 18

Polyoxometalate Oxidation of Phenolic Lignin Models

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A new environmentally friendly technology for wood pulp delignification, based on the use of polyoxometalates (POMs) and oxygen, is being developed. Polyoxometalate solutions that selectively oxidize lignin under anaerobic conditions can be reoxidized by oxygen and also catalyze the aerobic mineralization of the lignin fragments to CO_2 and H_2O, making possible the closed-mill manufacture of paper. Model studies on the oxidation of various lignin-like soluble compounds with POMs suggest that single-electron oxidation of phenolic substructures occurs first, followed by the hydrolysis of cationic intermediates. Detailed physico-chemical studies of the solutions of the alkali-metal salts of POMs show a correlation between the reduction potentials of POMs and the rate of oxidation of a phenolic lignin model that is ascribed to ion pairing between alkali-metal cations and POM anions.

The delignification of wood is a major process in the manufacturing of paper, one of the largest industries in the world. The goal of the delignification process is to chemically remove lignin (the component of wood that in residual quantities deteriorates the quality of paper) and to leave cellulose (the desirable component of wood) undamaged. The search for selective and environmentally friendly pulp bleaching technologies that can be used to achieve effluent free (closed-mill) manufacturing is a current challenge for the paper industry worldwide. The use of polyoxometalates (POMs) as reusable oxidants to achieve these goals was first reported in 1996 in (*1*). The chemical selectivity and economic feasibility of the newly developed process of pulp delignification by POMs makes it quite competitive with conventional chlorine-based and with alternative totally chlorine free (TCF) delignification technologies (*2-5*).

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Polyoxometalates

Polyoxometalates are structurally diverse anionic clusters consisting of d^0 metal cations, particularly W(VI), Mo(VI), V(V) and Nb(V) and oxygen anions arranged in MO_6 octahedral units (*6,7*). The attributes of POMs such as low cost, commercial availability, and synthetic tractability (acidity, solubility, thermal stability and redox potentials can be controlled) make them attractive for the use as acid or oxidation catalysts (*8-10*). One of the best studied POM families is Keggin-type heteropolyoxoanions of general formula $XM'_aM''_{12-a}O_b^{m-}$ where X^{n+} is a d- or p- block "heteroatom", M′ and M″ are d^n and d^0 metal centers, respectively (Figure 1). They have prominent advantages that can be attractive for oxidation reactions including delignification processes. First, many physical properties such as redox potential, solubility or molecular charge can be controlled by the choice of synthetic precursors or reaction conditions. Second, they can be reversibly reduced by one or more electrons. Third, the d^0 metal cations of parent POMs can be substituted by other d metal ions thus affording a multitude of POMs that exhibit a wide range of physical and chemical properties.

POM Delignification

Delignification technology based on POM catalysts is being developed at the Forest Products Laboratory of the U.S. Department of Agriculture, Forest Service, the Emory University Department of Chemistry and the University of Wisconsin-Madison, Department of Chemical Engineering. The overall wood pulp bleaching process is accomplished in 2 steps. First, unbleached pulp is heated in an aqueous solution of a fully oxidized POM (POM_{ox}) under anaerobic conditions to yield soluble oxidized lignin fragments ($Lignin_{ox}$) and the reduced POM (POM_{red}) (Scheme 1, Step 1). The bleached cellulose is then separated and the reduced bleaching liquor is treated with O_2 at elevated temperature and pressure (Scheme 1, Step 2). During this POM reoxidation step, the POM simultaneously catalyzes the O_2 oxidation of the dissolved lignin fragments to

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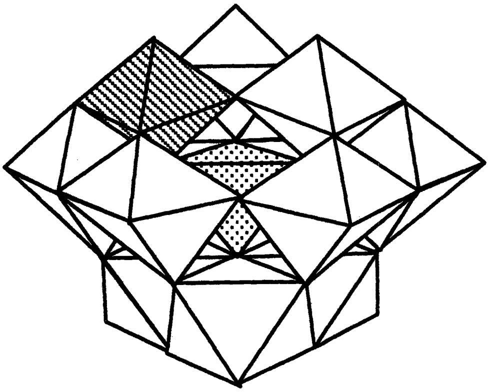
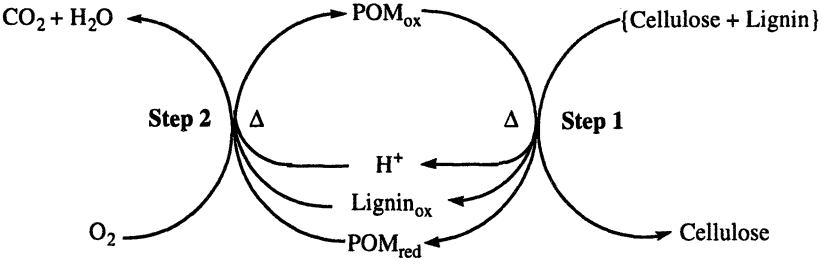


Figure 1. Polyhedral representation of monosubstituted Keggin-type POM $[X^{n+}VW_{11}O_{40}]^{(8-n)-}$ where the central tetrahedron represents the XO_4 ($X^{n+} = Al^{3+}$, Si^{4+} or P^{5+}) unit, the shaded octahedron represents the VO_6 unit and the unshaded octahedra represent the WO_6 units.

CO_2 and H_2O (aerobic mineralization). Thus, the net reaction for POM-mediated delignification is the selective transfer of electrons from lignin to O_2. The POM must possess a reduction potential positive enough to effectively oxidize lignin in Step 1 and sufficiently negative for spontaneous (i.e., thermodynamically favorable) reoxidation by O_2 in Step 2.



Scheme 1

Of the many POMs formulated and evaluated for effectiveness in delignification (Step 1), $[SiV^{V}W_{11}O_{40}]^{5-}$ (**1**) is among the most selective (*3*). For effectiveness in the overall cycle (Scheme 1), the POM must be reoxidized by O_2 (Step 2). Though thermodynamically favorable, the reoxidation of reduced **1**, $[SiV^{IV}W_{11}O_{40}]^{6-}$ ($\mathbf{1_{red}}$), is extremely slow on an industrial time scale even at 200 °C and several atmospheres of oxygen. While new equilibrating POM systems designed for optimum effectiveness in both Steps 1 and 2 have been developed (the unique and complex self-buffering chemistry of these new systems will be reported elsewhere), **1** remains an ideal starting point for detailed mechanistic study of POM delignification (*11,13*).

In this chapter, reactions of **1** are used to address mechanistic aspects of oxidative delignification by POMs (Step 1). Studies included here include the investigation of the reactions of **1** (chosen for quantitative lignin-model studies as it is not only very effective in delignification, but also readily available in chemically and isomerically pure form) with lignin model compounds, and physico-chemical studies of several isostructural POMs and correlations between their thermodynamic (reduction potentials) and kinetic (reactivity with a phenolic lignin model) properties.

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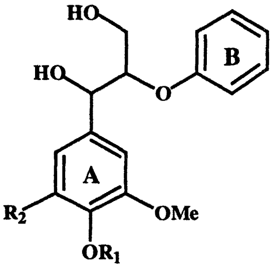
Results and Discussion

Oxidation of Lignin Models by Polyoxometalates

Lignin has a polymeric structure consisting of cross-linked hydroxylated (ca. 15%) and methoxylated phenylpropane monomers. In kraft lignin, the amount of hydroxylated phenyl structures increases to ca. 20-40 % as a result of the cleavage of aryl ether bonds during kraft pulping (*12*). Preliminary studies of the oxidation of various lignin models, reported in a recent publication (*3*), showed that phenolic substructures are substrates for oxidation by **1**. Under actual delignification conditions (pH 7 phosphate buffer, 3 h at 125 °C), the phenolic β-aryl ether lignin dimer model **3** undergoes a rapid oxidative cleavage by **1**. Oxidation of the non-phenolic analogue **4** is under investigation.



3 ($R_1 = H, R_2 = OMe$)
4 ($R_1 = Me, R_2 = OMe$)
5 ($R_1 = H, R_2 = H$)

Further studies of the oxidation of lignin-like phenolic compounds with no *ortho* substituents (i.e., R_2 = H) indicate that phenoxyl radicals are likely intermediates (*13*). At room temperature, the oxidation of **5** by **1** results in the rapid oligomerization of **5**, a result consistent with coupling of phenoxyl radical intermediates. Phenoxyl radicals are also very likely intermediates in the delignification of pulp; relative to solutions, however, radical coupling is less likely in media of restricted molecular mobility, such as within the ligninocellulosic matrix of the cell wall.

In order to study the cleavage reaction of a phenolic model under the conditions wherein competing oligomerization processes are minimized, the substituted phenolic model, **3**, was used. At room temperature, **3** was rapidly oxidized by **1** to yield an approximately 1:1:1 mixture of **6**, **7** and **8** (Scheme 2).

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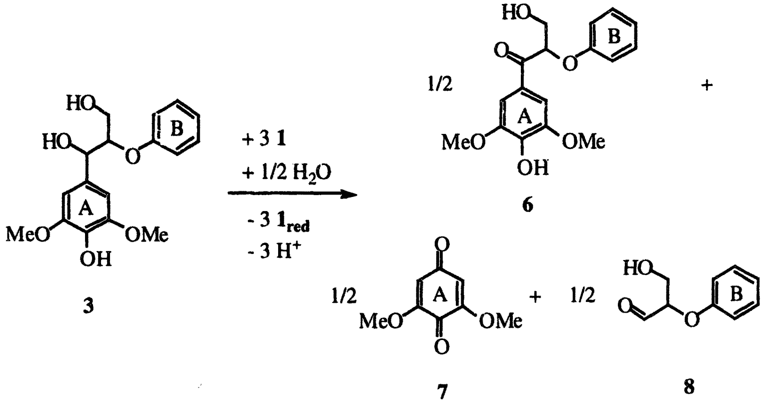
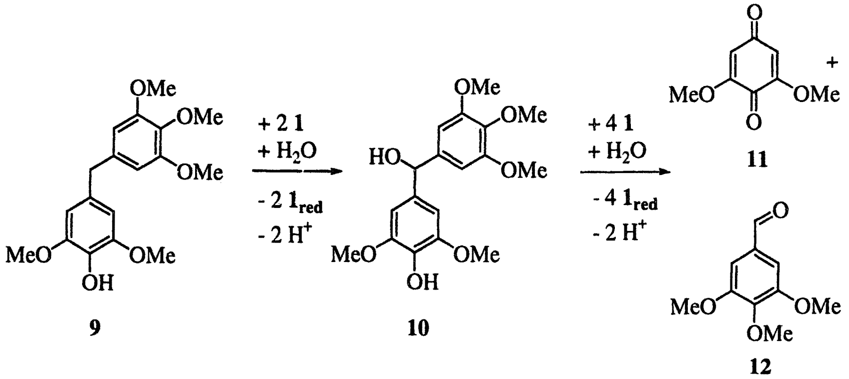

3
+ 3 1
+ 1/2 H_2O
- 3 1_{red}
- 3 H^+
1/2
6
+
1/2
7
+ 1/2
8
HO
MeO
OMe
OH
O
A
B

Scheme 2

The α-ketone, **6**, results from dehydrogenation of the benzylic hydroxyl group of **3**, while compounds **7** and **8** result from repeated oxidation and hydrolysis of **3** and subsequent intermediates. To give a 1:1 ratio of two-electron (**6**) and four-electron (**7** and **8**) oxidation products, three equivalents of **1** would be consumed per equivalent of **3**. This was confirmed experimentally.

The phenolic diphenylmethane, **9** (Scheme 3), which is believed to form under alkaline pulping conditions, rapidly reacts with **1** at room temperature. The alcohol **10**, along with small amounts of *para*-benzoquinone, **11**, and


9
+ 2 1
+ H_2O
- 2 1_{red}
- 2 H^+
10
+ 4 1
+ H_2O
- 4 1_{red}
- 2 H^+
11
+
12
HO
MeO
OMe
OH
O

Scheme 3

3,4,5-trimethoxybenzaldehyde, **12**, were found among the reaction products. After reaction at elevated temperature, the alcohol, **10**, a possible intermediate in further oxidative cleavage, was not detected.

These results strongly suggest that phenol oxidation reactions are crucial in delignification of native and residual kraft lignins by **1**. Oxidation reactions of non-phenolic lignin components remain under investigation. Experimental details of the results summarized in Schemes 2 and 3 are reported in ref. (13).

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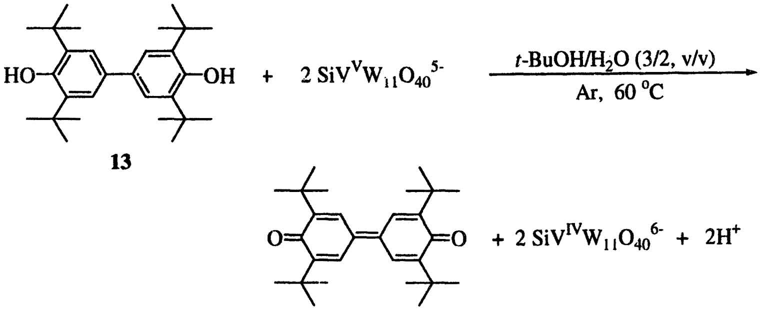
Oxidation of a Phenolic Lignin Model by a POM. Kinetics Studies.

While investigation of the oxidation of the solubilized lignin models under homogeneous conditions does not allow for assessment of mass-transfer limitations that may be operable during reactions in wood fibers, it does provide considerable information about the redox and fragmentation chemistry. However, the dimeric lignin-like models described above are difficult to use for detailed kinetic (mechanistic) studies of their oxidations by **1** because the reactions give rise to product distributions. As noted above, unsubstituted phenols are prone to couple upon one-electron oxidation. Moreover, other functional groups (-CHOH, $-CH_2-$, etc.) can also undergo oxidation. Thus, the ideal model for studying oxidation of the phenolic moiety should possess a reactive phenolic group attached to a substituted phenyl ring that is otherwise resistant to oxidation by **1**. Of the many compounds evaluated for their suitability in detailed kinetic studies, 3,3′,4,4′-tetra-*tert*-butylbiphenyl-1,1′-diol (**13**, diphenol, abbreviated $(PhO)_2H_2$) was found to optimally satisfy these requirements. This diphenol reacts with $Li_5SiV^VW_{11}O_{40}$ (Li_5**1**) in *tert*-butanol/water (3:2, v/v) at 60 °C under argon to give a single organic product 3,3′,4,4′-tetra-*tert*-butyldiphenoquinone,**14**, and $SiV^{IV}W_{11}O_{40}^{6-}$ ($\mathbf{1}_{red}$), by the stoichiometry shown in eq 1. One equivalent of **14** (quantified by ^1H NMR) and 2 equivalents of $\mathbf{1}_{red}$ (determined by UV-vis spectroscopy) are formed per equivalent of **13** consumed. During the reaction, 2 equivalents of H^+ should be generated; therefore, the aqueous component of the solvent mixture was buffered with 0.1 M LiOAc/ HOAc (pH 4.76) prior to mixing with *tert*-butanol.

The reaction kinetics were monitored using UV-vis spectroscopy by following the characteristic absorbance of $\mathbf{1}_{red}$ ($\lambda = 520$ nm, $\varepsilon = 619$ L mol^{-1} cm^{-1}). The rate law determined by the initial rate method, $d[\mathbf{1}_{red}]/dt = k_{app}[(PhO)_2H_2][\mathbf{1}]$, is first order in both substrate (**13**, $(PhO)_2H_2$) and oxidant (**1**) and effectively zero order in both $\mathbf{1}_{red}$ and acetate anion. These results suggest that the rate limiting step (r.l.s.) is bimolecular, the forward reaction for the reduction of **1** is much faster than the reverse one under the reaction conditions

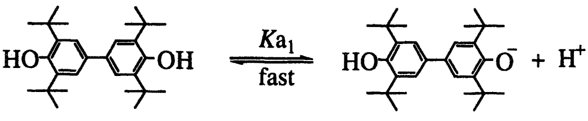


(1)

used, and subsequent proton abstraction by acetate (acting as a base), if it occurs, is significantly faster than the r.l.s.

The reaction rate is independent of ionic strength, but dependent on the Li^+ concentration. Therefore, when the amounts of **1**, **1**$_{red}$ or LiOAc were varied in kinetic studies, the concentrations of the alkali-metal cations were carefully controlled by adding the corresponding amounts of LiCl in order to keep the Li^+ concentration constant. The dependence on Li^+ concentration is attributed to ion pairing between **1** and Li^+. Ion pairing in polyoxometalate solutions is discussed in the next section and also addressed in recent work (*14,15*).

The apparent second-order rate constant, determined as $k_{app} = (d[\mathbf{1}_{red}]/dt)_o / ([(PhO)_2H_2]_o [\mathbf{1}]_o)$, is dependent on the proton concentration (Figure 2). The observed data indicate parallel pathways for the reduction of **1**. Such a complex dependence of the reaction rate on $[H^+]$ is characteristic for phenol oxidation reactions in aqueous or mixed solutions where both a phenol molecule and a phenolate ion are in equilibrium with each other and compete for the oxidant (*16-18*). Under the reaction conditions used in this study, the protonated diphenol molecules $(PhO)_2H_2$ are the dominant species (in *tert*-butanol/water (3/2, v/v) at 60 °C, pKa_1 and pKa_2 of the diphenol were estimated to be ca. 13.5-14.5 and 14.0-15.0, respectively). Oxidations of fully protonated $(PhO)_2H_2$ and of monoprotonated $(PhO)_2H^-$ both contribute to the reaction rate, while oxidation of fully deprotonated $(PhO)_2^{2-}$ is not kinetically significant (see Figure 2, inset, and discussion below). A mechanism for the phenol and monophenolate oxidation by **1** consistent with the data is given in eqs 2-5.



(2)

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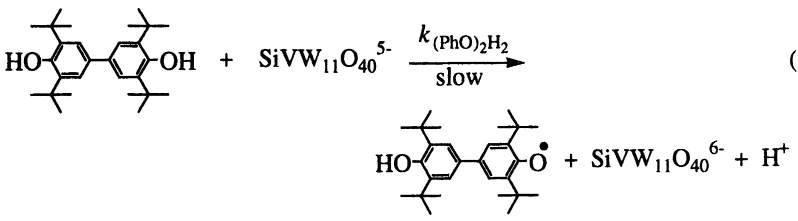
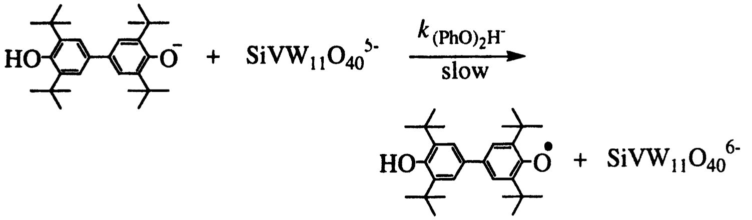
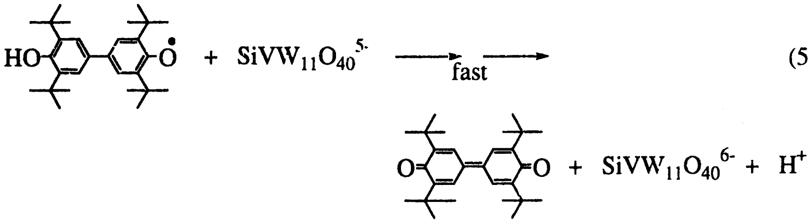


(3)



(4)



(5)

Under these reaction conditions, **1** is a single-electron oxidant; therefore both the diphenol and the monophenolate are likely to react with **1** in parallel pathways (eqs 3 and 4) to give phenoxyl radicals, the initial one-electron oxidation intermediates. Given that steps 3 and 4 are rate limiting, the steady-state approximation applied to the concentrations of the intermediates can be used to derive the dependence of the reaction rate on the concentrations of the reactants (eq. 6):

$$\frac{d[\mathbf{1}_{red}]}{dt} = 2\left(k_{(PhO)_2H_2} + \frac{k_{(PhO)_2H^-}Ka_1}{[H^+]} \right)[\mathbf{1}][(PhO)_2H_2]_{total} \qquad (6)$$

The derived rate expression, eq 6, is the same as the empirical rate law with the apparent rate constant $k_{app} = 2\,(k(PhO)_2H_2) + k((PhO)_2H^-) \bullet Ka_1/[H^+])$, which validates the approximations made. Rigorously, including Ka_2, $k_{app} = 2\,(k(PhO)_2H_2) + k((PhO)_2H^-) \bullet Ka_1/[H^+] + k((PhO)_2^{2-}) \bullet Ka_1 \bullet Ka_2/[H^+]^2)$. However, the plot of k_{app} *vs.* $1/[H^+]$ (Figure 2, inset) gives a straight line rather than a parabolic curve, which demonstrates that the third term (due to oxidation of the di-anion, $(PhO)_2^{2-}$) is not kinetically significant. From the linearized function (k_{app} *vs.* $1/[H^+]$), $k((PhO)_2H_2)$ and the product of $k((PhO)_2H^-)$ and Ka_1 are obtained. Thus, the reactivity of the monophenolate towards **1** ($k((PhO)_2H^-)$ = ca. 10^5-10^6 L mol^{-1} s^{-1}) is roughly 10^7-10^8 times higher than that of the diphenol ($k((PhO)_2H_2) = 0.02$ L mol^{-1} s^{-1}).

The empirical rate law (eq 6), established only for the initial rates, can be extrapolated up to 90-95 % conversion of phenol by using the integrated dependence of the absorbance *vs.* time (eq 7), where ε is the extinction coefficient of $\mathbf{1}_{red}$, $[(PhO)_2H_2]_o$ and $[\mathbf{1}]_o$ are initial concentrations of the diphenol and **1**, and A_t and A_o are the initial absorbance and the absorbance at time *t*, respectively, and *l* is the pathlength.

$$A_t = \varepsilon\left\{[1]_o - \frac{[(PhO)_2H_2]_o - \frac{[1]_o}{2}}{\frac{[(PhO)_2H_2]_o}{[1]_o}\exp\left[k_{app}\left([(PhO)_2H_2]_o - \frac{[1]_o}{2}\right)t\right] - \frac{1}{2}}\right\}l + A_o \qquad (7)$$

Eq 7 is the Lambert-Beer's law $A_t = \varepsilon[\mathbf{1}_{red}]_t l + A_o$ where the expression for the concentration of $\mathbf{1}_{red}$ at time *t* was obtained by integration of the differential equation $d[\mathbf{1}_{red}]/dt = k_{app}[(PhO)_2H_2][\mathbf{1}]$ (empirical rate law) given the stoichiometry $[(PhO)_2H_2]:[\mathbf{1}] = 1:2$ (eq 1).

The experimental data were fitted to eq 7 by varying the k_{app} values in order to minimize the sum of the squares of the deviations. From Figure 3, one can see that the model holds up to 90-95 % conversion of diphenol, after which point the steady-state approximation itself is no longer valid. Thus, the proposed mechanism (eqs 2-5) is valid for nearly the entire reaction.

In order to establish the more intimate mechanistic details of the reaction, kinetic isotope studies with the diphenol deuterated in OH-positions were carried out. The deuterated diphenol ($(PhO)_2D_2$) was generated *in situ* by using the deuterated solvent mixture *tert*-BuOD/D_2O (pD of the D_2O component = 3.90). The reactions were run at low pH(pD) to eliminate the contribution of the monophenolate oxidation in the apparent rate constant. The determined k_H/k_D value of 1.2 ± 0.2 indicates that hydrogen atom transfer is unlikely to occur in the activation complex.

The possible mechanisms are narrowed further by determination of the activation parameters associated with the rate-limiting step. From the plot of $\ln(k((PhO)_2H_2)/T)$ vs. T^1 (50-85 °C), the enthalpy of activation ($\Delta H^\ddagger$) and the entropy of activation ($\Delta S^\ddagger$) were found to be 8.5 ± 1.4 kcal mol^{-1} and -39 ± 5 esu, respectively. The low $\Delta H^\ddagger$ is characteristic of outer-sphere electron transfer reactions. The negative $\Delta S^\ddagger$ value is consistent with a bimolecular r.l.s. (eq 3). The absolute value of the entropy of activation is unusually high for an outer-

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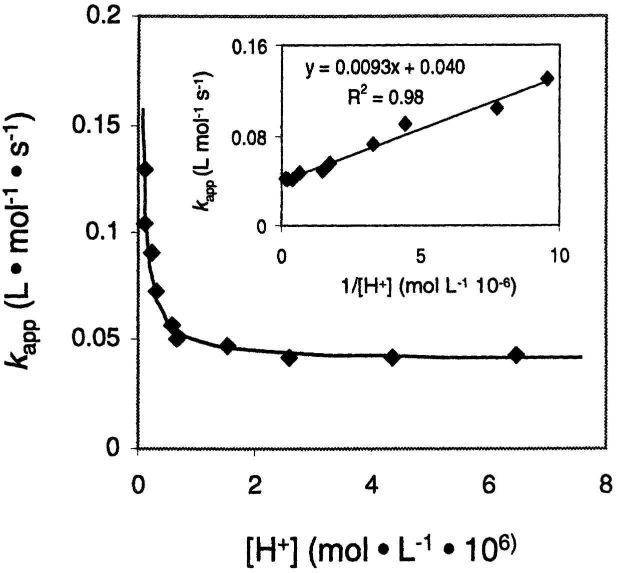

Figure 2. Plot of k_{app} vs. $[H^+]$; $[H^+]_{obs} = 6.5 \bullet 10^{-6} - 1.3 \bullet 10^{-7}$ (measured in tert-butanol/water mixture), $[Li_5 1] = 0.48$ mM, [PhOH] = 2.86 mM, [LiOAc] = 0.1 M, [HOAc] = 0.1 M. 60 °C. Inset: plot of k_{app} vs. $1/[H^+]$.

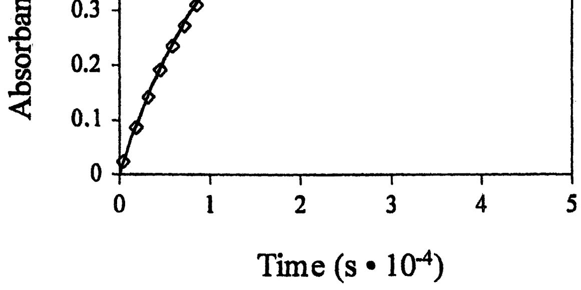

Figure 3. Absorbance at 520 nm as a function of reaction time for the oxidation of diphenol by $Li_5 1$ (open circles) and kinetic model fitting to eq 7 (solid line); $k_{app} = 0.0412$ L mol^{-1} s^{-1}, sum of standardized square residuals = $4.4 \bullet 10^{-4}$.

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sphere electron transfer process, but can be rationalized by a sterically restricted activated complex. The steric constraint arises from the very low ratio of the size of the C-OH and V^V=O moieties relative to the total surface area of the two large reacting molecules, diphenol and **1** (ca. 0.002 and 0.0004, respectively). That is, for electron transfer to occur, the reacting species must collide in a very specific and restricted orientation involving the close proximity of the OH-group and the V^V=O moiety of **1**, which greatly increases the entropy of activation.

These detailed kinetic data indicate that this diphenolic lignin model is oxidized by **1** *via* an outer-sphere single-electron transfer mechanism. Highly reactive phenoxyl radicals are likely generated in the bimolecular rate-limiting step. At high pH, the parallel pathway involving oxidation of the monophenolate anion, that is significantly more reactive than the parent phenol, contributes to the overall rate. Clearly, under actual delignification conditions (water and neutral or basic pH), oxidation of phenolic substructures is likely to involve reactions of both protonated and deprotonated phenolic groups.

Ion Pairing in POM Solutions

As discussed above, the reaction rate increases with an increase in alkali-metal cation concentration (Figure 4). Detailed kinetic and physico-chemical studies of this phenomenon show that in *tert*-butanol/water (3:2, v/v), 1:1 ion pairing between **1** and Li^+ occurs (*14,15*).

$$\mathrm{Li^+} + \underset{\mathbf{1}}{\mathrm{SiVW_{11}O_{40}}^{5-}} \xrightleftharpoons{K_{IP}} \underset{\mathbf{Li1}}{\{(\mathrm{Li^+})\mathrm{SiVW_{11}O_{40}}\}^{4-}} \tag{8}$$

Both unpaired and paired POM anions (**1** and Li**1**) are believed to react with the diphenol in parallel pathways (eqs 9 and 10).

$$\mathbf{1} + \mathrm{(PhO)_2H_2} \xrightarrow{k_0} \mathbf{1}_{\mathbf{red}} + \mathrm{(PhO)_2H^{\bullet}} + \mathrm{H^+} \tag{9}$$

$$\mathrm{Li}\mathbf{1} + \mathrm{(PhO)_2H_2} \xrightarrow{k_1} \mathrm{Li}\mathbf{1}_{\mathbf{red}} + \mathrm{(PhO)_2H^{\bullet}} + \mathrm{H^+} \tag{10}$$

Based on the modified reaction mechanism (eqs 8-10), the functional dependence of the apparent rate constant (k_{app}) on Li^+ concentration was derived:

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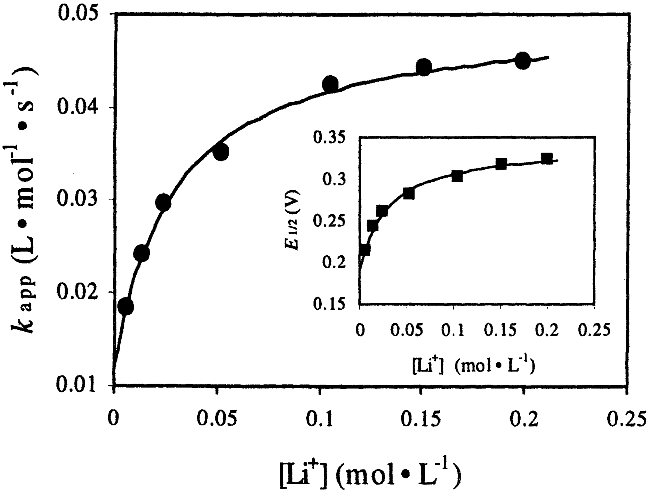

Figure 4. Plot of k_{app} vs. [Li^+]; [Li^+] = [LiCl] + 5[$Li_5$1] + [LiOAc], [LiCl] = 0.0 - 0.2 M, [$Li_5$1] = 1.0 mM, [PhOH] = 2.0 mM, [LiOAc] = 1 mM, [HOAc] = 1 mM, 60 °C, argon. Inset: plot of $E_{1/2}$ vs. [Li^+], same conditions except no PhOH present; $E_{1/2}$ vs. Ag/AgCl electrode. The solid lines are the best fits to eqs 11 and 12, sum of standardized square residuals = $6.4 \cdot 10^{-5}$.

$$k_{\mathrm{app}} = \frac{k_o + k_1 K_{\mathrm{IP}}[\mathrm{Li}^+]}{1 + K_{\mathrm{IP}}[\mathrm{Li}^+]} \qquad (11)$$

where k_o and k_1 are the rate constants for the oxidation of the diphenol with unpaired and paired POM anions, respectively, and K_{IP} is an ion pair formation constant. Experimental rate data were successfully fitted to the model (eq 11) by non-linear least square regression (three parameters, k_o, k_1 are K_{IP}) were allowed to vary to minimize the sum of squares of the deviations). A similar functional dependence of the observed reduction potentials of **1** on Li^+ concentration was also observed (Figure 4, inset). Under the same experimental conditions as those used in the kinetic studies, the curve in Figure 4, representing the functional dependence of the weighted-mean reduction potential, E, on $[\mathrm{Li}^+]$, reaches a plateau. Moreover, the kinetic and electrochemical data in Figure 4 were both *simultaneously* fit to the corresponding model equations 11 and 12 by varying the parameters K_{IP} (identical in both eqs 11 and 12), k_o, k_1, E_o and E_1.

$$E = \frac{E_o + E_1 K_{\mathrm{IP}}[\mathrm{Li}^+]}{1 + K_{\mathrm{IP}}[\mathrm{Li}^+]} \qquad (12)$$

The excellent fits ($K_{\mathrm{IP}} = 34$ L mol^{-1}, $k_o = 0.012$ L mol^{-1} s^{-1}, $k_1 = 0.050$ L mol^{-1} s^{-1}, $E_o = 0.19$ V, $E_1 = 0.34$ V) shown in Figure 4 support the proposed mechanism involving 1:1 ion pairs (eqs. 8-10).

Three independent lines of evidence in support of the 1:1 ion-pair stoichiometry shown in eqs 8 and 10 were obtained (*15*). Further kinetic and electrochemical studies of ion-pairing with various alkali-metal cations (K^+, Na^+ and Li^+) and the POM anions $\mathrm{PVW_{11}O_{40}}^{4-}$, $\mathrm{SiVW_{11}O_{40}}^{5-}$ and $\mathrm{AlVW_{11}O_{40}}^{6-}$ demonstrate similar rectangular hyperbolic dependencies of rate constants and reduction potentials on alkali-metal cation concentrations (*14,15*). Both the reduction potential of each ion pair (E_1) and the rate constant (k_1) for its reduction by the diphenol increase in the order: $\mathrm{Li}^+ < \mathrm{Na}^+ < \mathrm{K}^+$. This trend is rationalized in terms of the relative sizes of the ion pairs. The effective hydrodynamic radii (r) of the ion pairs were calculated using the Stokes-Einstein equation: $D = kT/6\pi\eta r$ where diffusion coefficients (D) were determined by chronoamperometry, and solvent viscosity (η) was measured by the capillary-flow method. For the ion pairs $\{(\mathrm{M}^+)\mathrm{SiVW_{11}O_{40}}\}^{4-}$ in *tert*-butanol/water (3:2, v/v) at 60 °C, the effective radii were found to decrease in the order: $\mathrm{M}^+ = \mathrm{Li}^+ > \mathrm{Na}^+ > \mathrm{K}^+$ (8.3, 7.7 and 6.8 Å, respectively; standard deviation ± 4 %). As the effective crystallographic radii of hexacoordinated alkali-metal cations increase in the order: $\mathrm{Li}^+ < \mathrm{Na}^+ < \mathrm{K}^+$ (*19*), the charge densities and hence solvated radii of these ions decrease in the same order (*20*). Thus, the alkali-metal cations with

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larger atomic numbers have smaller solvated radii and form more intimate ion pairs, which posses larger electron affinities.

These discussed results establish that alkali-metal cations and POM anions can form association complexes (ion pairs) in mixed organic – aqueous solutions and that ion-pair formation accelerates phenol oxidation. Ion pairing also occurs in pure water (used in POM delignification) at high concentrations of POMs and/or alkali metal salts. Additional studies on ion association in aqueous POM solutions are needed to quantify these associations.

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Experimental

Materials. The potassium-salts of **1** and $\mathbf{1_{red}}$ were synthesized according to the literature method (*21*). The lithium- and sodium-salts of **1** were prepared from the corresponding potassium-salt by cation exchange chromatography using Amberlite IR-120 (plus) ion exchange resin. The preparation of lignin models **3**-**5** and **9** has been described (*13*). The diphenol **13** (99% purity) was used as received from Polysciences, Inc. All other materials were reagent grade.

Methods. Kinetics data were collected using a Hewlett Packard 8451A diode array spectrometer equipped with both a thermostat (± 0.1 °C) and a stirrer. Quartz cuvettes equipped with Schlenk-type tops were used to degas the solutions prior to reaction. Cyclic voltammetry experiments were carried out under argon using a BAS CV-50W voltammetric analyzer. A three electrode cell with a glassy carbon working electrode, a platinum auxiliary electrode and a Ag/AgCl (3M NaCl) reference electrode was used. Non-linear square fits were performed using the Solver Function in Microsoft Excel-98.

Conclusions

The phenolic lignin substructures, modeled by lignin-like dimer compounds, were found to be reactive substrates towards oxidative cleavage by **1**. Under actual delignification conditions, outer-sphere oxidation of both phenols and phenolates is likely to occur. Alkali-metal cation – POM anion association in solution increases the electron affinity of POMs and accelerates the rate of phenol oxidation

Acknowledgments

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Chapter 19

Polyoxometalate-Based Closed Systems for Oxidative Delignification of Wood Pulp Fibers

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A new technology based on the use of polyoxometalates (POMs) as delignification agents is under development. These reagents are chlorine free and can be used under conditions wherein they oxidize lignin and chromophores in wood pulp fibers while leaving the cellulose undamaged. Their promise is enhanced by the fact that they can be reactivated with oxygen, in a separate stage, under conditions that result in oxidation of the organic byproducts of the delignification process. Thus, they can be continuously recycled in closed systems that can provide the basis for a new class of closed mill technologies in which the consumable oxidant is oxygen, and the primary byproducts are carbon dioxide and water. Such systems are inherently the product of well-integrated subsystems, each of which accomplishes a specific task. The two key tasks that have been the points of focus for the program are delignification of kraft pulps by POM solutions under anaerobic conditions, and regeneration of the oxidative capacity of spent POM liquors simultaneously with oxidation of the organic byproducts to water and carbon dioxide. The first group of POMs investigated provided the basis for establishing the requirements for each of the tasks. They led to demonstration of selective delignification and effective re-oxidation and mineralization. The current generation of POMs have been optimized for effectiveness in both tasks. Furthermore they are readily synthesized and they are inherently self-buffering. Their performance in the context of traditional bleaching is indicated by the capacity to reduce the kappa levels of softwood kraft pulps from 30 to below 5 while retaining viscosity above 20. More recently it has been shown that they can effectively delignify kraft pulps at much higher kappa levels in the 100 to 110 range, and soda AQ pulps at kappa levels about 120. Thus POM delignification technology is applicable both for achieving TCF closed mill systems in the traditional bleaching context and, perhaps more significantly, for cost effective incremental expansions of the capacity of recovery-boiler-limited pulp mills.

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Introduction

The polyoxometalates (POMs) are a class of delignification agents that promise to provide the basis for a new class of closed-mill delignification technologies. The POMs used as delignification agents are transition-metal-substituted cluster ions similar in structure to many mineral ores. They are entirely chlorine and sulfur free and can be used under conditions that make them very selective in their action on the constituents of lignocellulosics. They can be designed to be highly selective oxidants that can be targeted to the substructures that occur in lignin without also attacking the polysaccharide components of the wood fibers; in their active states, and when applied under anaerobic conditions, they can oxidize lignin and related chromophores, while leaving the polysaccharides undamaged. Their action on lignin results in reduction of the POMs so that their oxidative capacity has to be regenerated before they can be recycled. This is done in a separate stage wherein the reduced POMs are exposed to oxygen at elevated temperatures. The conditions for re-oxidation also result in oxidation of the organic byproducts of delignification. Thus, POM delignification is similar to kraft pulping processes in that the organic by products are oxidized rather than released to the environment; the POMs can be continuously recycled in a closed system. For these reasons, the POMs provide the basis for a new class of closed-mill delignification technologies in which the consumable oxidant is oxygen, and the primary byproducts are carbon dioxide and water. While the POMs were initially developed in the search for alternatives to chlorine based bleaching technologies, it has now become apparent that they can also complement kraft pulp mills by reducing the organic load on the recovery boilers. Thus they also present opportunities for expanding the capacity of kraft pulp mills with relatively low levels of capital expenditure. Eventually, together with soda-anthraquinone pulping, they may make possible entirely sulfur free pulping systems.

We have previously reported on different generations of POMs that were investigated in our laboratory, and assessed them with respect to their suitability for use in economically viable and environmentally benign delignification systems (1-11). Here we present an overview of the evolution of closed-system delignification by POMs and the advances that were the basis for optimization. We also report on recent breakthroughs that have led to POMs that combine the most attractive characteristics of the POMs that were investigated in earlier stages. Because of these advances, we are now in a position to move toward development of pilot-scale facilities for the use of POMs in delignification of commercial pulps.

We would note here that a number of other investigations of the POM-based delignification have been reported (12), but they have focused on the use of POMs to promote oxidative delignification in a catalytic manner. As they do not address closed-system operation, we will not include them in our discussion.

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Background

The delignifying action of POMs was discovered as part of a new program on alternative pulping and bleaching technologies specifically directed at finding more selective oxidative systems. Selectivity was chosen as the point of emphasis because it was known that wood-degrading fungi use highly selective peroxidase enzymes to degrade lignin. These enzymes leave the cellulose intact so that it can later be hydrolyzed to glucose by a system of hydrolases; the fungi can then directly assimilate it as a nutrient. An interim goal of our program therefore, was duplication of the action of the peroxidase enzymes, but in inorganic systems that are useable in an industrial context.

We first recognized that the selectivity of the peroxidases is based on the way in which they use transition-metal ions in controlled environments to catalyze the oxidation of lignin. There is, in fact, a considerable literature on organic peroxidase analogs that are effective in selective oxidation of lignin; for example (13, 14). These systems, however, remain only of academic interest because they require complex organic platforms for the transition-metal ions, and the organic substructures are not stable at temperatures necessary to achieve the high rates required for an industrial process.

The search turned to inorganic systems that can mimic the action of the peroxidases but have the stability prerequisite for application at elevated temperatures. It had earlier been reported that POMs provide controlled environments for transition metal ions, not unlike those provided by the porphyrins (15,16). They, therefore, seemed the logical candidates as a point of departure for our efforts. We recognized that polyoxometalate cluster ions provide a ligand environment that can mimic the function of the organic platform in many fungal enzyme systems. Our program thus began with the goal of simulating the action of enzymes, with the level of selectivity that we were seeking, by placing active metal ions in POM structures.

The POMs are a class of oligomeric, metal-ion oxide clusters. The ones we have used in our program are among the most common and have structures identified as the Keggin type. In such structures, which are approximately spherical clusters, the anions typically include 12 structural transition-metal atoms, such as tungsten or molybdenum, surrounding a main-group atom, such as phosphorous, silicon, or aluminum; in most instances approximately 40 oxygen atoms are integrated into the structure. To make them active for delignification, one or two of the structural metal atoms of the cluster ion are replaced with a first-row transition-metal atom; the ones we have used most frequently are vanadium and manganese.

In order to achieve the high degree of selectivity, the POMs have to be applied to the pulp in stoichiometeric amounts and under anaerobic conditions. It would have been preferable if the POMs could have been used catalytically, however, the re-oxidation of POMs by oxygen is accompanied by the generation of free radicals that are not selective in their action on substrates. Absent a component in the system that can act as a free radical scavenger, the re-oxidation of the POMs in the presence of pulp fibers can result in significant degradation of the polysaccharides. We have observed this both directly (17, 18) and indirectly (19). We have compared the application of POMs to pulp under oxygen with similar application under nitrogen. Application under oxygen resulted in significant reduction of pulp viscosity beyond that which can be attributed to hydrolysis. The

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degree of viscosity reduction due to hydrolysis can be estimated from the loss when the POMs are applied under nitrogen (19).

In the approach we have adopted, the re-oxidation of the POMs for recycling is carried out in a separate stage, after their separation from the pulps. In this stage, the spent delignification liquors are exposed to oxygen at elevated temperatures. The generation of free radicals during this stage has become the basis for mineralizing the lignin fragments dissolved in the liquor during delignification. The conditions are such that much of the organic material is oxidized to carbon dioxide and water; the POMs are stable since the structural metal ions in the clusters are in their highest oxidation states and the POM preparation is in a state of equilibrium (20). In the equilibrium state, a number of isomeric POM species coexist with oligomeric forms of the constitutive metal oxides. After re-oxidation the POM solution is recycled to delignifiy pulp again.

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The two steps of the process can be schematically represented in the following equations:

Delignification:

$$\mathrm{LigH_2 + POM_{ox} \longrightarrow Lig_{ox} + POM_{red} + 2H^+} \qquad (1)$$

Reoxidation and Catalytic Mineralization:

$$\mathrm{Lig_{ox} + POM_{red} + 2H^+ + nO_2 \longrightarrow POM_{ox} + mCO_2 + pH_2O} \qquad (2)$$

When they are combined in a cyclic process, they can be represented as in Figure 1, where it is more clearly obvious that the two steps may be integrated into a two-stage cyclic process.

The polysaccharides are not included in any of these representations as, apart from hydrolytic cleavage, they are not susceptible to oxidation under the conditions of unit operation **A**.

In Figure 1, the delignification operation (A) is represented as the reaction of the POM, in its oxidized forms, with the lignin to produce the soluble oxidized lignin and the POM in its reduced form. The solubilized oxidized lignin, together with the reduced POM, is then fed into the re-oxidation stage, where the POM is re-oxidized and the solubilized lignin is mineralized.

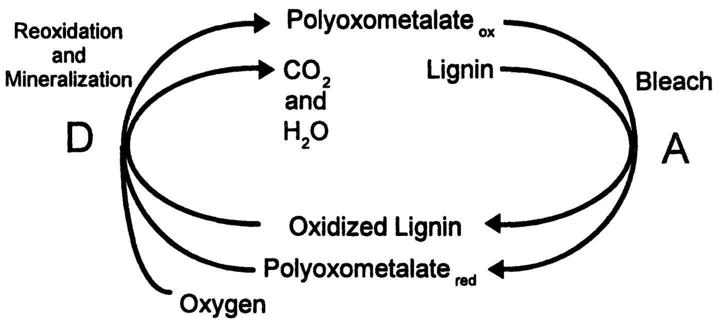

*Figure 1. Net reactions of the POM process. Unit operation A denotes the delignifcation reactor under anaerobic conditions and unit operation **D** represents the wet-oxidation reactor.*

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Further examination of equations 1 and 2 above reveals another important consideration that arises when the two steps are to be coupled. It is a consequence of the release of protons during the oxidation of lignin and their consumption during the re-oxidation by oxygen; the protons are incorporated into the H_2O formed by the reduction of the oxygen. For the coupling of these two stages, it is therefore necessary to have a buffer system. Yet another advantage of the POMs is that they are constituted from metal oxides that can occur in oligomeric forms in equilibrium with the Keggin anions and that can, through reorganization of the oligomeric distributions, provide the necessary buffering capacity. This will be illustrated below in the results of cyclic studies of delignification and re-oxidation using one of the POM systems investigated.

Process Concept

For use of the POMs in a delignification process that can be scaled up for commercial use, it is necessary to consider how the different chemical transformations can be organized in relation to each other. At the heart of the process, there are the two complementary operations indicated by the equations and schematic shown above. In the first one, an aqueous solution of POM in its oxidized form is applied to the pulp under anaerobic conditions. It oxidizes and solublilizes the lignin in the pulp and is itself reduced. When its oxidizing capacity has declined to a point determined by overall process parameters, the spent liquor, with all the soluble organics released during the delignification operation, is separated from the pulp. In the second operation, the spent solution of POM and organics is oxidized with oxygen at an elevated temperature. Under these conditions, the reduced POM is re-oxidized, and simultaneously, it catalyzes the oxidative degradation of the soluble organics to carbon dioxide and water.

The process envisioned for POM delignification is shown in Figure 2. As noted earlier, the key unit operations are delignification in stage **A** and wet oxidation in stage **D**. Commercial installations will also require a number of additional complementary unit operations. The operation **B** in Figure 2 designates the stages required for separating the pulp from the spent POM liquors. It begins with pressing to remove as much of the POM and soluble organic liquid phase as is feasible. The next stage is the washing of the pulp. It has been demonstrated that POMs can be effectively separated from pulp by washing. The high negative charge of the POM anions makes separation from the negatively charged pulp fibers quite easy. Preliminary laboratory studies have shown that the washing operation is such that it lends itself well to the use of commercially available washing equipment.

Operation **C** then represents concentration of the POM and soluble organics separated from the pulp during washing. Here again, evaporation of the wash water can be readily accomplished in commercially available evaporator systems. It is also anticipated that a stage for removal of non-process elements will be incorporated into the overall scheme. Since multiple preliminary studies have demonstrated that operations **B** and **C**, representing washing and recovery, can be accomplished with established technologies, the primary focus of our program has been on improvement of the delignification (**A**) and wet-oxidation stages (**D**) and on simplification of the processes for synthesis of the POMs. We are also seeking a deeper understanding of the physical and chemical processes that are the rate limiting steps in both **A** and **D** stages.

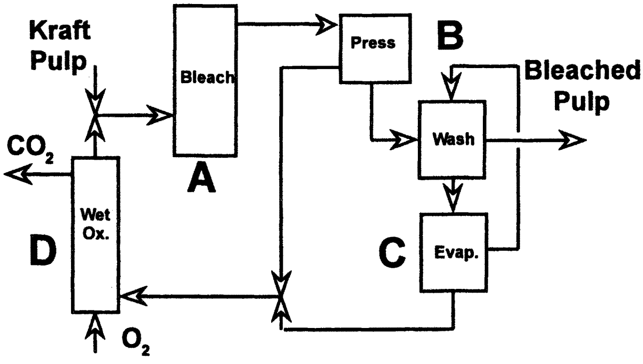

Figure 2. A simplified schematic representation of the POM delignification process.

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Evolution of POM Development

Early Studies

In our first reports on POM-based delignification, we described successful operation in a low-consistency, multistage sequence at 125⁰C, using $Na_5PV_2Mo_{10}O_{40}$ as the POM (4,5). Hereinafter it will be designated as PV_2Mo_{10}, and the same notation will be used in describing other POMs, as in most instances the counterion is Na and the complement of O atoms is similar. The work with PV_2Mo_{10} was important in that it allowed us to demonstrate the high degree of selectivity of the oxidation that can be designed into POMs and it revealed to us their self buffering capacity. However, it is not stable at pH levels above 4, so that it could not be used at temperature levels that result in commercially feasible delignification rates. At such temperatures and pH levels, the hydrolysis of the cellulose occurred at unacceptable rates. It should be noted here that, unless otherwise noted, the pulps used in all trials were northern pine kraft from a commercial source.

The next cycle of delignification trials were carried out with $Na_5SiVW_{11}O_{40}$, henceforth denoted by $SiVW_{11}$, which is stable at neutral pH levels and has a slightly higher oxidation potential than PV_2Mo_{10}. It was possible to reduce the duration of the individual stages and to operate successfully at temperatures below 100⁰C. We also demonstrated effective operation at medium consistency (11%). Two representative examples are described.

In the first, carried out at 125⁰C, the kappa number was reduced from 24.5 to 12.6 with a 30-minute POM stage, followed by caustic extraction. The corresponding loss in viscosity was from 28.7 to 22.2 m Pa s. This was accomplished by application of 0.5 M $SiVW_{11}$ in 1.0 M phosphate buffer, which kept the pH in the 6 – 7 range.

In the second example, carried out at 90⁰C, two successive POM stages, followed by caustic extraction, reduced the kappa number from 24.5 to 11.9, with a corresponding decline in viscosity from 28.7 to 27.3 m Pa.s. This was accomplished by application of 0.5 M $SiVW_{11}$ in 0.5 M acetate buffer, which kep the pH at 5.5. The caustic extraction between the two stages utilized 1.0 % NaOH for 2 hours at 85⁰C and 10 % consistency. These two examples pointed to the possibility of further optimization of POM-based delignification.

It was with the $SiVW_{11}$ system, as well, that the possibility of achieving very good paper properties was first demonstrated. The measured paper properties that were demonstrated with $SiVW_{11}$ are shown in Table I, where

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they are compared with properties of the same pulp after a $C/D_{30}E$ treatment and also with the properties of a control sample that was subjected to the same sequence of treatments as the POM-treated pulp, but without the presence of the POM; the control had a final kappa of 20.2 (9,10,11).

The conditions of application of the $SiVW_{11}$ differed somewhat from those described above in that the concentrations of the POM solutions were lower so that more than one application was necessary to fully accomplish the oxidation of the ligning. The POM delignified pulps the properties of which are reported in Table I were produced by applying 0.05 M $SiVW_{11}$ in 0.2 M phosphate buffer. The first application was for 1 hour; this was followed by two 2 hour applications; all were at 125°C at 3 % consistency. The caustic extraction was with 1 % NaOH for 2 hours at 85°C at a consistenty of 2%.

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Table I. Paper Properties of POM Delignified Pulps

	$C/D_{30}E$	POM	Control
Viscosity (mPa sec)	23.1	19.5	23.2
Burst Index (kN/g)	8.65	8.18	7.11
Tear Index (mN m^2/g)	9.03	9.30	9.45
Tensile Index (N m/g)	109.0	103.7	96.5
Zero-Span (N m/g)	160.8	148.4	150.3
Density (kg/m^3)	796.1	792.3	769.9

- 5000 rev. PFI mill (360 ml CSF)
- Initial Kappa of 24
- $C/D_{30}E$ and POM bleached to a Kappa of 4.6

It is clear that the POM-treated pulp has properties that are comparable to those of the $C/D_{30}E$ pulp. The Achilles heel of the $SiVW_{11}$ system was, however, that it is not as easily re-oxidized with O_2 at elevated temperatures, at least not at rates that were regarded as desirable in a commercial operation.

We have reported that wet-oxidation can be carried out effectively and POM-containing liquors can be recycled for delignification (5). These wet oxidation studies, using PV_2Mo_{10}, demonstrated the feasibility of achieving a low level of chemical oxygen demand (COD), together with complete re-oxidation of the POM. They also demonstrated that the resulting liquors, with the residual COD, can be used for delignification and are as effective as the fresh POM solutions. Although PV_2Mo_{10} is not a feasible delignifcation agent from a commercial perspective, it remains an important POM in studies directed at characterizing the delignification liquors and the chemical transformations that occur during wet oxidation. It is anticipated that the results of these studies will be helpful in facilitating further optimization of the wet oxidation process.

Although the first generation of POMs did not provide the basis for a commercial process, it did allow us to explore the behavior of POMs in the context of delignification and wet oxidation. The key to successful commercialization was the identification of POMs that are effective in both delignification and wet oxidation stages, and that are also self-buffering.

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The Second Generation

The development of the second generation of POMs was based on combining the characteristics of the two earlier systems. It was found that by shifting to variations on the $SiVW_{11}$ we could move towards better performance. We report here the development of a new group of POMs that have a number of advantages over the first generation. In addition to being well suited to both delignification and wet oxidation, the new POMs are stable at pH levels above neutral, so hydrolysis of the cellulose is significantly reduced. Another important advance associated with the introduction of this new group of POMs has been the development of a new synthetic procedure that results in an equilibrium composition, which is inherently stable and, therefore, can be recycled repeatedly in a closed system (20). The new synthetic procedure is much more simple than traditional synthetic approaches. The availability of this new procedure should facilitate the design of POM production processes on an industrial scale, and it should make research in the field of POM delignification more accessible to other laboratories.

A number of new POMs, all of which appear to be stable above pH 7 and are re-oxidized by oxygen, have been explored on the basis of the new synthetic approach. These include SiV_2W_{10}, $AlVW_{11}$, and a group that is described by the formula $SiV_xMoW_{11-x}O_{40}$. Here it should be noted that the formula presented is intended to reflect the molar ratios in the final equilibrium mixture, which is likely to include a number of different Keggin structures, the majority of the isomers of the dominant species.

The POM that has been studied most extensively is SiV_2W_{10}, which has been routinely used to reduce kappa number from about 32 to below 10 under several different conditions, including multiple stages at 10%

consistency. The yield levels, in most instances, are above 95%. The effectiveness of this POM is illustrated in Figure 3, which shows the results of a cyclic experiment wherein the same solution of SiV_2W_{10} was used repeatedly first to delignify pulp fibers and then as the vehicle for mineralization of the organics removed as it was re-oxidized prior to re-use.

In the experiment shown in Figure 3, the same 0.5M solution of $SiV_2W_{10}O_{40}$ was used again and again, to delignify softwood kraft pulp from kappa of 33 to an average kappa of 8, and then re-oxidized with oxygen. The conditions of application were at a consistency of 3 %, for three hours at 150°C. The average drop in viscosity was from 30.4 to 19.4 and the average yield was 94.9 %.

The experiment was repeated for twenty cycles; each cycle in the figure represents one bleaching reaction and one re-oxidation reaction. The left scale shows the % reduction of the POM, while the right scale shows the pH at the end of each cycle. The even oscillation of the reduction curve shows that the POM can be fully regenerated and used effectively in delignification over an indefinite number of cycles. The shifting of the pH within a very narrow band demonstrates the effectiveness of the POM as a self-buffering system.

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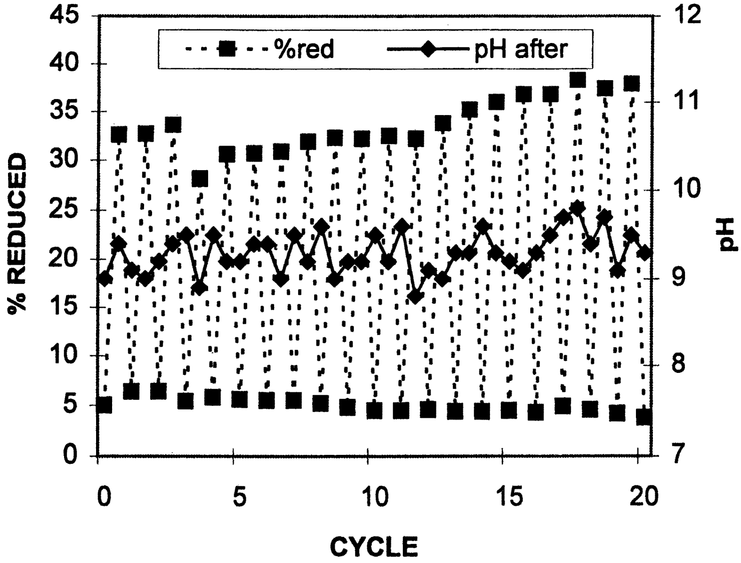

Figure 3. The results of cyclic experiments of successive delignification and oxidation reactions carried out with SiV_2W_{10}. Left scale: % reduction of the POM. Right scale: pH.

When one considers that a proton is released into solution for each POM molecule reduced, it can be estimated that the pH of the solution would have dropped to 1 during the delignification reaction if theself-buffering capacity of the system were not avaialble. Of course, significant decline of the pH would and resulted in unacceptable degrees of hydrolytic degradation of the cellulose in the pulp fibers. The buffering capacity of the POM solutions is the result of shifting of the balance between the condensation and hydrolysis reactions of the oligomeric constitutive metal oxides present in the equilibrium solutions with the POMs (20).

In addition to its extensive use in tests with pulps at kappa levels in the low 30s, this POM has been used to test the feasibility of applying POM technology to higher kappa pulps. This possibility is of interest in mills that have well-established delignification and bleaching stages but could benefit from increasing the kappa level at which the pulp is removed from the digesters. The results of some trials, with a southern pine linerboard pulp at a kappa of 65, are shown in Table II. It is clear that even if the POM stage was used to completely delignify the pulp, the viscosities are still acceptable.

Beyond the studies with SiV_2W_{10}, other variations on the $SiVW_{11}$ system have been explored. It has been shown that some are even more effective in meeting the criteria for commercial development. In particular it has been observed that addition of small amounts of molybdenum, as in $SiV_{0.9}MoW_{10.1}$, can enhance the effectiveness to the point where the properties of the pulps match those of commercial ECF pulps at acceptable residence times in the delignification reactor. It has also been demonstrated that $SiV_{0.9}Mn_{0.1}MoW_{10.1}$ is even more effective in the regeneration and mineralization steps during the wet-oxidation stage.

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Table II: Delignification of Southern Pine Kaft Linerboard with 0.5 M SiV2W10

Temp (°C)	Time (hr.)	Liquor to wood (mL/g)	Final pH	Final Kappa[1]	Final Viscosity (mPa·s)	Yield on Pulp (%)	Yield on Wood [2] (%)
145	7	100	9.3	7	27	90	50
145	14	100	9.3	2	18	90	50

[1] initial kappa 65

[2] estimated pulping yield of 55% on OD wood

Conclusion

It is now clear that POM-based technology has the potential to outperform currently available technologies with respect to facilitating closed-mill systems and reducing environmental impact. It has also been shown that the technology is more energy efficient and quite competitive economically. The member companies participating in the consortium supporting this work, jointly with the USDA Forest Service and the U.S. Department of Energy, are now considering the establishment of a pilot facility to demonstrate the commercial feasibility of POM-based delignification technologies.

Acknowledgments

We wish to acknowledge support of this work by the USDA Forest Service, the U.S. Department of Energy, Office of Industrial Technologies, and the member companies of the Polyoxometalate Bleaching Consortium.

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Chapter 20

Lignin Degradation Reactions in Aerobic Delignification Catalyzed by Heteropolyanion $[PMo_7V_5O_{40}]^{8-}$

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The fundamental principles and the mechanisms of lignin oxidative degradation in the reaction system $[PMo_7V_5O_{40}]^{8-}/O_2$ are reviewed. The delignification was shown to follow a complex mechanism including heterolytic and homolytic oxidative stages with the catalyst. In addition, minor contributions of acidolysis and autooxidation reactions have been noted. The phenolic lignin structural units are involved in oxidation with $[PMo_7V_5O_{40}]^{8-}$ and VO_2^+ ions dissociated from the parent heteropolyanion. The oxidation rate of non-phenolic structures is 5-7 times lower than that of phenolic ones. The influence of heterogeneous character of the catalysis on the mechanisms of lignin oxidation is discussed.

Recent trends for highly selective lignin oxidation reactions with dioxygen include the application of metal-oxygen anion clusters (polyoxometalates) as regenerable redox reagents (*1,2*) or as catalysts (*3,4*). The principles of oxidative delignification with polyoxometalates (POMs) have been widely discussed previously (*1-4*) and can be formulated briefly as follows: the POMs, having an energetic barrier to lignin oxidation lower than that of dioxygen, oxidizes the lignin and then, the reduced form of the POM is re-oxidized by O_2 (Fig. 1). The final products of the lignin oxidation and the oxygen reduction are

In Oxidative Delignification Chemistry; Argyropoulos, D.;
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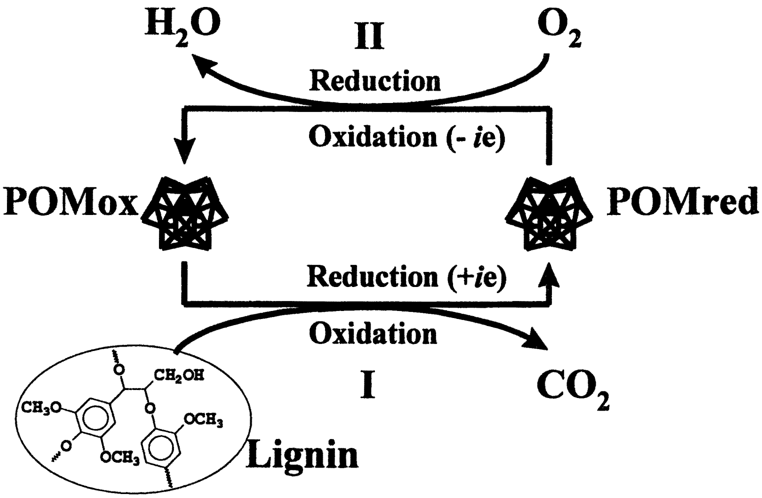

Figure 1. General scheme of POM catalyzed oxygen delignification (POMs are presented as Keggin compounds in polyhedral-filling representation).

carbon dioxide and water, respectively. The oxidation of lignin and re-oxidation of the POMs can occur in one stage (aerobic oxidation of lignin in the presence of POM) or in separate stages (step I as shown in Fig. 1 is performed under anaerobic conditions). In the first case POMs may be considered as oxidative catalysts. POMs catalyzed oxygen delignification was successfully used both for the pulping and bleaching needs (*3-7*). Due to the complete oxidation of organic matter and because bleaching liquors can be continuously re-used, POM based oxidation processes are considered as a very promising approach towards Total Effluent Free (TEF) bleaching plants (*1,5*). Between a large variety of POMs, the heptamolybdopentavanadophosphate heteropolyanion (HPA-5) with general formula $[PMo_7V_5O_{40}]^{8-}$ is particularly adapted for the catalytic purposes mentioned above (*3-7*).

The aim of this paper was to review the fundamentals of the oxidation catalysis with HPA-5 and to summarize the results of latest investigations of the lignin degradation mechanisms in the reaction system HPA-5/O_2.

Heteropolyanion $[\mathrm{PMo7V5O40}]^{8-}$ as a Redox Catalyst

The nomenclature and structural varieties of POMs are well described elsewhere (*8*). POMs are composed primarily by early transition metal cations in their high oxidation states and oxoanions with a variety of structures.

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Particularly, heteropolyanions (HPA) are soluble salts of polyoxoanions having the general formula $[X_xM_mO_y]^{q-}$, where X is a heteroatom (X=P, Si, B, etc.) and M is an addenda atom (M= W^{IV}, V^{V}, Mo^{VI}, etc.). HPA of T_d symmetry, quasi-spherical structures derived from assemblies of twelve MO_6 octahedra around a tetrahedron containing a heteroatom, are known as Keggin compounds (Fig. 2). The heteropolyanion $[PMo_7V_5O_{40}]^{8-}$ (HPA-5) belongs to Keggin type HPA-n (where **n** is the number of vanadium atoms in HPA) series $[PMo_{12-n}V_nO_{40}]^{(3+n)-}$. The main structural features and redox properties of HPA-5 are similar to those of others HPA-n (n=2,3,4,6) and were extensively studied in last decades (*9,10*).

HPA-n (n=2-6) are well known highly selective catalysts in oxidative organic syntheses due to their easily re-oxidation by oxygen even at room temperatures (*9,10*). The basic reversible redox reactions involved in organic electron donor's oxidation with HPA-n may be shown as follow (*9*):

$$\text{Substrate} + [HPA]_{ox} + mH^+ \leftrightarrow Hm[HPA]_{red} + \text{oxidized substrate} \qquad (1)$$

$$H_m\,[HPA]_{red} + m/4\;O_2 \leftrightarrow [HPA]_{ox} + m/2\;H_2O \qquad (2)$$

where $[HPA]_{ox}$ and $[HPA]_{red}$ represent, respectively, the oxidized and reduced forms of heteropolyanions. Actually, Eq. 1 and Eq. 2 describes the steps **II** and **I**, respectively, in the catalytic oxidative delignification (Fig. 1). In the aerobic oxidation the electron donor organic substrates are oxidized by vanadium atom

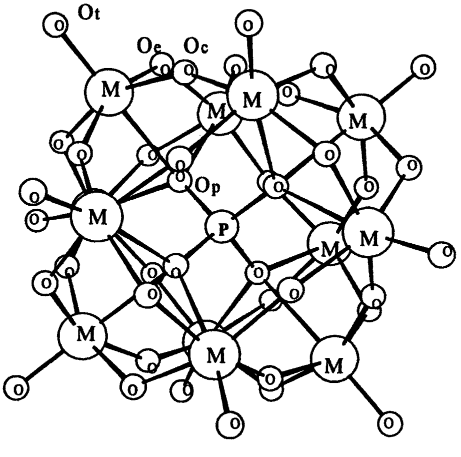

Figure 2. The Keggin structure of $[PM_{12}O_{40}]^{3-}$ polyanion (α-isomer) in ball and stick representation (central PO_4 tetrahedron is surrounded by 12 MO_6 octaedra; terminal (O_t),corner-sharing (O_c), edge-sharing (O_e) and phosphorous-linked (O_p) oxygen atoms and the metal atoms (primarily W, Mo, V) are depicted by small and big colorless balls, respectively.

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V (V) in the HPA-n composition (Eq. (1)). The reduced HPA-n containing V (IV) are re-oxidized by dioxygen (Eq. (2)). Hence, it is the $V^{5+} \leftrightarrow V^{4+}$ transformation that is responsible for the redox properties of HPA-n (*9*). The thermodynamic conditions for the organic substrate (as the lignin) oxidation under aerobic conditions can be formulated as follows:

$$E^{o}_{substrate} < E^{o}_{HPA\text{-}n} < E^{o}O_2\,(1.23\text{ V at pH }1) \qquad (3)$$

The reported redox potentials of HPA-n (n=1-6) vary from +0.60 to 0.72 V (pH 1) depending on the number of V atoms in the HPA composition (*11*). HPA-1 has a maximum E° value that decreases in the order: HPA-1>HPA-2> ...HPA-6 (*11*). HPA-n are normally stable in solutions at pH 2.5-5 and temperatures up to 250-300 °C. The instability of HPA-n at pH>5.5 determines their application only under acidic conditions. The increase of the number of V atoms in the composition of HPA-n deteriorates their structural stability at the same pH of solutions. In the acidic solutions (pH ≤ 3) HPA-5, like others HPA-n, can disproportionate to give species with the number of V atoms different than that of the parent HPA-5:

$$2\text{HPA-5} \leftrightarrow \text{HPA-(5-x)} + \text{HPA-(5+x)} \qquad (4)$$

The hydrolytic dissociation of HPA-5 occurs through the formation of so-called lacunary derivatives of the parent Keggin compound (Fig. 3), obtained by removal of one or more (up to three) V groups:

$$\text{HPA-5} \leftrightarrow \text{HPA-(5-x)} + x\text{VO}_2^{+} \qquad (5)$$

It was shown, based on ^{51}V, ^{31}P NMR and ESR data, that HPA-5 in acidic (pH ≤ 3) solutions represents a complex mixture of different structural isomers of HPA-n (n=1-5) and VO_2^+ ions (*4*). The equilibrium between all the mentioned species depends on the nature of the solvent (aqueous solution or solution

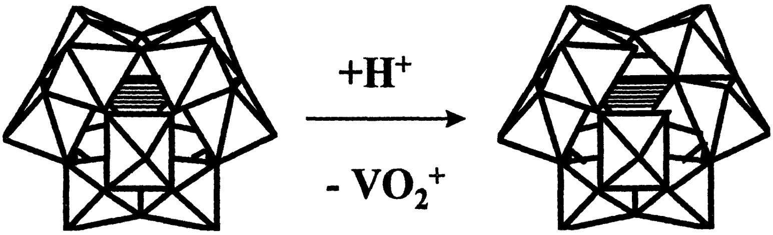

Figure 3. Polyhedral-filling representation of α-$PM_{12-x}V_xO_{40}$ Keggin polyanion (left) and corresponding lacunary structure (right) derived from dissociation of one V group.

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containing organic solvent), pH, the extent of the reduction of HPA-5 and its concentration (*4*).

The VO_2^+ ions produced via HPA-5 hydrolytic dissociation (Eq.5) have higher redox potential (about +0.90 V at pH 1 (*11*)) than that of HPA-n (n=1-5), simultaneously presenting in the acidic solution. This fact plays a key role in the oxidative delignification since VO_2^+ ions were considered as the main active species in the catalytic oxidation of lignin (*4*). Thus, both monomeric VO_2^+ cations and HPA-n participate in the lignin oxidation (step **I**, Fig. 1).

The mechanisms of oxidation of electron-donor organic substrates (EDOS) with HPA-n are not completely disclosed. Discussions on this matter can be found in recently published reviews (*9,10,12*). Most of the investigators suggest an outer-sphere electron transfer mechanism between the substrate and the oxidized HPA through the formation of an intermediate complex [Substrate...HPAox] involving corner or terminal oxygen atoms of the HPA-n (*9,10,13*). The oxidation of EDOS with HPA-n (n ≥2) can occur via multielectron-transfer (particularly, via two consecutive one-electron oxidations) mechanism (*9,10*).

As far as the mechanisms of O_2 reduction by HPA-n are concerned, both outer-sphere (*14*) and inner-sphere (*13*) mechanisms have been proposed. According to the prevalent view, inner-sphere mechanisms involves intermediate complexes between O_2 and V(IV) addendum atoms of the HPA through the breaking of a V(IV)-O bond between the reduced (d^1) vanadium ion and a bridging μ–oxygen atom (*12*).

Mechanisms of HPA-5 Catalyzed Oxygen Delignification

Reaction scheme of the oxygen delignification catalyzed by HPA-5. The lignin oxidation mechanisms in aerobic oxidation with HPA-5 (pH 2-2.5) have been elucidated through delignification kinetic studies (*4*), structural investigations on the residual and dissolved lignins (*15*), analysis of the low-molecular-weight oxidation products (*15*) and on reactions with lignin model compounds (*16,17*). The simplified reaction scheme can be summarized as follows:

$$L + V(V) \rightarrow L^\cdot + V(IV) + H^+ \quad (6)$$

$$L^\cdot + V(V) + H_2O \rightarrow \text{depolymerized lignin} + V(IV) + H^+ \quad (7)$$

$$L^\cdot + L^\cdot \rightarrow L\text{-}L \quad (8)$$

$$L^\cdot + O_2 \rightarrow L\text{-}OO^\cdot \quad (9)$$

$$L\text{-}OO^\cdot + V(IV) \rightarrow L\text{-}OO^- + V(V) \quad (9')$$

$$L\text{-}OO^- \rightarrow \text{low-molecular-weight products} \quad (9'')$$

The rate-determing step of the oxidative delignification is one-electron oxidation (Eq. 6) of the lignin (L) by V(V), mostly as VO_2^+ ions released from the HPA-5, but also in the composition of HPA-n (n <5) (Eq. 5). The second electron abstraction from the lignin aromatic group (Eq. 7) leads to the formation of a cyclohexadienyl cation followed by the hydrolytic cleavage of alkyl-phenyl or aryl-OMe bonds (demethoxylation). The oxidation steps 6 and 7 presented above are reliable for the phenolic structural units. The oxidation of non-phenolic units proceeds by different manner and will be discussed later. Radical coupling of one-electron oxidized structural units leads to lignin "condensation" (Eq. 8). These coupling reactions result in a strong negative effect on the delignificaion at moderate temperatures (< 60 °C). Reactions 9, 9′, 9′′reflect the suggested participation of dioxygen in lignin autooxidation, which is almost suppressed by V(IV) in the HPA-n composition or as VO^{2+} ions (Eq. 9′).

The reactivity of phenolic lignin units in the reaction system HPA-5/O_2. The lignin phenolic units are mostly involved in the oxidation with HPA-5 (*16,17*). The reactivity of lignin structural units in step 6 follows from the thermodynamic conditions of the oxidation with HPA-n (Eq. 3). The lignin units possessing higher electron-donor character are easier to react with the catalyst. This explains why syringyl type units are more readily oxidized by HPA-5 than the guaiacyl type lignin units (*15,16*). The relative reactivity of different phenolic lignin structures was estimated based on analysis of lignin oxidation products (*15*) and on results of voltammetric experiments with monomeric model compounds (*16*). The facility of oxidation of phenolic lignin structures was suggested to decrease in the order: hydroxybenzyl structures > benzylether structures > α-carbonyl structures (*16*). According to the voltammetric studies in aqueous solutions of HPA-5 (pH 2) and monomeric model compounds it was proposed that both HPA-n (n < 5) and VO_2^+ ions can oxidize phenolic lignin units (*16*).

The mechanism of oxidative cleavage of β-O-4 linkages in phenolic structures has been studied using 1-(3-methoxy-4-hydroxyphenyl)-2-(2-methoxyphenoxy)ethanol (**A**) as a lignin model compound (*17*). The main reaction pathways suggested, based on the analysis of the products obtained (**III-VII**), are shown in Fig. 4. Two consecutive one-electron oxidations of the aromatic ring with the catalyst (both VO_2^+ and HPA-n) are followed by hydrolytic degradation of the formed cyclohexadienyl cation **Y**. This reaction intermediate was confirmed using Electrospray Ionisation Mass Spectrometry (ESI/MS) applied in the positive mode (*17*). The heterolytic cleavage of alkyl-phenyl bonds was also suggested to occur in the oxygen delignification of wood catalyzed by HPA-5 (*15*).

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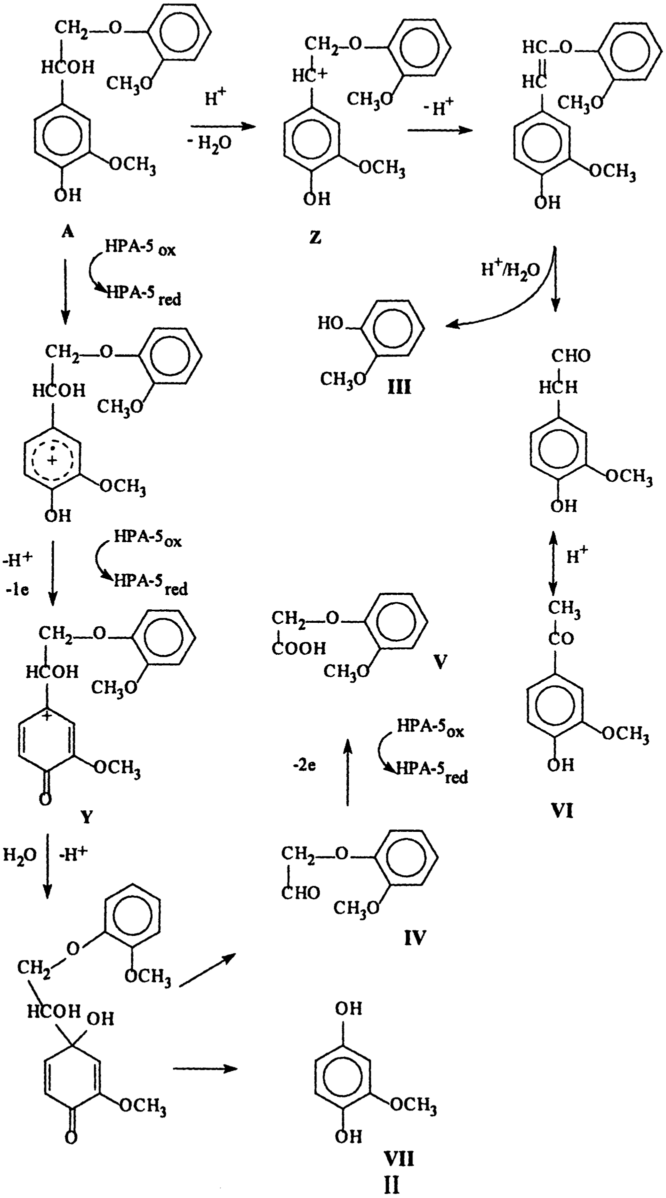
A
Z
H+
- H2O
-H+
H+/H2O
HPA-5 ox
HPA-5 red
-H+
-1e
Y
H2O
-H+
III
CHO
HCH
H+
VI
V
-2e
IV
VII
II
OCH3
OH
CH3O
HCOH
CH2—O

Figure 4. The reaction scheme of dimer A oxidation in the reaction system HPA-5/O_2 (90 °C; 20 min.; C_{dimer}= 8 mmol/l; $C_{HPA\text{-}5}$= 2 mmol/l, P_{O2}= 0.5 MPa) (ref. 17).

The occurrence of acid-catalyzed (acidolytic) cleavage of β-O-4 linkages was concluded based on appearance of acetoguaiacone **(VI)** as a typical acidolysis product (Fig. 4). The formation of benzyl cation **Z** (Fig. 4), a known intermediate of the acidolytic degradation, was detected by means of ESI/MS (*17*). The occurence of acid-catalyzed degradation of lignin was additionally concluded from the study of eucalyptus wood oxygen delignification catalyzed by HPA-5 in acidic ethanol-water solutions (*15*). However, the contribution of acidolysis in the cleavage of β-aryl ether bonds of lignin is much lower than that inserted by oxidative degradation with HPA-5: the delignification degree increased five times with addition of HPA-5 at the same conditions (100 °C, 5h, oxygen pressure 0.5 MPa; pH 2) of the oxygen pulping as compared to the control experiment without HPA-5 (*3*).

Since the catalytic oxidative delignification with HPA-5 is performed under acidic conditions (pH 2-3) and elevated temperatures (80-100 °C), the lignin condensation reactions take place slowing the delignification rates (*3,15*). The condensation reactions are especially important in the case of guaiacyl type lignin (softwood delignification). The addition of organic solvents to the pulping/bleaching liquor prevents substantially the undesirable condensation reactions thus promoting the delignification (*3,15*).

Reactivity of non-phenolic lignin units in the reaction system HPA-5/O_2. Non-phenolic lignin structural units are hard to oxidize in the reaction system HPA-5/O_2. However, their contribution in the lignin oxidative degradation is not negligible (*16*). The comparison of the oxidation extent of vanillyl and veratryl alcohols, as phenolic and non-phenolic lignin model compounds, respectively, under the same reaction conditions (90 °C; 40 min; P^oO_2= 0.5 Mpa) showed a conversion of veratryl alcohol 5-6 times lower than that of vanillyl alcohol (*16*). VO_2^+ ions were suggested to be the active species in the oxidation of non-phenolic model compounds.

The reaction mechanism of the oxidative cleavage of β-O-4 linkages in non-phenolic lignin structures was elucidated using a dimeric model compound 1-(3,4–dimethoxyphenyl)-2-(2-methoxyphenoxy)ethanol **(B)** (*17*). Based on the identified oxidation products **(III, VII, X-XIII)**, the homolytic cleavage of β-O-4 and Cα-Cβ linkages in **B** were suggested as the main reaction routes (Fig. 5). The rate-limiting reaction step is one-electron electron oxidation of the guaiacyl group with the catalyst followed by the formation of the corresponding cation-radical (Fig.5). The formation of products such as vanillin **(XII)** and 3,4-dihydroxybenzoic acid **(XIII)** indicated the occurrence of the demethoxylation reactions. In general, the reaction pathways discovered were very close to those

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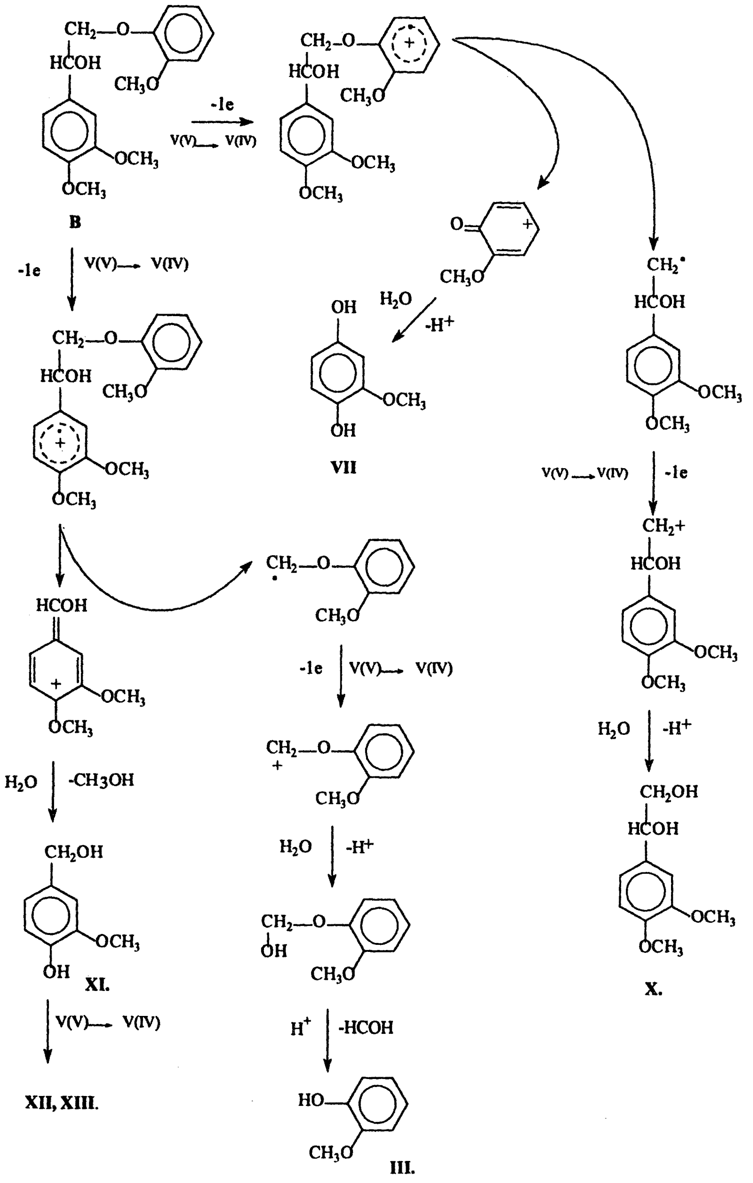
B
-1e
V(V) → V(IV)
VII
H₂O
-H⁺
X.
XI.
-CH3OH
XII, XIII.
-HCOH
III.
CH₂—O
HCOH
CH₃O
OCH₃
CH₂OH
HO

*Figure 5. The reaction scheme of dimer **B** oxidation in the reaction system HPA-5/O_2 (90 °C; 20 min.; C_{dimer}= 8 mmol/l; $C_{HPA\text{-}5}$= 2 mmol/l, P_{O2}= 0.5 MPa). V(V) and V(IV) are in the composition of VO_2^+ and VO^{2+} ions, respectively. (ref. 17).*

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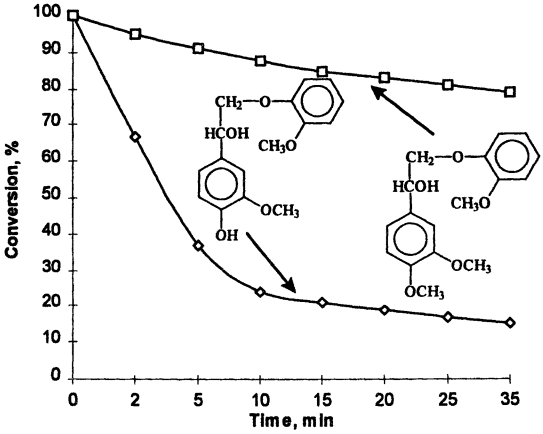

Figure 6. Kinetics of oxidative degradation of β-aryl ether dimeric model compounds. The rate constant (within first 5 min) of the phenolic dimer (1.9 · 10^{-1} min^{-1}) is about 7 times higher than that of the non-phenolic dimeric lignin model compound (2.6 · 10^{-2} min^{-1}). The reaction conditions were as follows: 80°C; C_{model}= 8 mmol/l; C_{HPA-5}= 2 mmol/l; $P^{o}O_2$= 5 · 10^{-3} MPa; solution acetonitril-water (30:70, v/v). The reaction mixtures were analyzed by HPLC: column - Lichrospher 100 RP18, 5μm 250 x 4 mm; eluent: acetonitrile-water (30:70 v/v); flow rate of 1ml/min; detector -UV-VIS Hitachi L4250 at 280 nm.

previously concluded to occur in the oxidation of non-phenolic arylglycerol β-arylether structures by LiP (*18*). The active catalytic species in the reaction system HPA-5/O_2 involved in the cleavage of β-O-4 structures were suggested to be VO_2^+ ions dissociated from the parent HPA-5 (*17*).

The kinetics of oxidative degradation of phenolic and non-phenolic β-aryl ether dimeric lignin model compounds in the glass flask under low oxygen pressure is presented in Fig. 6. A pseudo first-order reaction kinetic was observed with phenolic dimer within first 5 min of the oxidation followed by pseudo zero-order kinetics. Such an oxidation behavior is explained by HPA-5 re-oxidation problems with dioxygen at low pressures which, after the 5 min of the reaction, turns as a rate-limiting step of the model compounds oxidation. This is not a case for the oxidation in autoclave at pressures more than 0.2 MPa (*4*). The comparison of the rate constants of the oxidation (within first 5-min of the reaction) showed about 7 times faster oxidation of the phenolic dimeric lignin model compound as compared with the non-phenolic one (Fig. 6).

Lignin structural transformations in HPA-5 catalyzed oxygen delignification. In order to further clarify the lignin behavior in the reaction system HPA-5/O_2, a lignin sample dissolved from the wood tissue in the fast period of delignification, when it's still present in the form of high-molecular-weight compound, was used. Fine cuttings of eucalyptus wood were cooked to 46 % delignification degree in ethanol-water (50:50, v/v) solution at 100 °C, during 2.5 h and wood-to-liquor ratio 10 (pH 2, HPA-5 concentration 2 mmol/l; initial oxygen pressure 0,7 MPa). The reactor was quickly cooled and the waste solution separated from the wood solid residue. After the addition of water in excess the precipitated lignin was isolated by centrifugation and then was washed with distillate water and freeze-dried. The obtained oxidized lignin sample (EOL) and the sample of dioxane eucalyptus lignin (DL), selected as the model of the lignin in untreated eucalyptus wood, were analyzed by 1D ^{13}C NMR and 2D ^{1}H-^{13}C NMR techniques. The attribution of signals was made based on known databases (*19,20*).

The quantitative ^{13}C NMR spectra are presented in Fig. 7. The EOL showed the typical features for oxidized lignin such as the presence of aromatic and aliphatic carboxyl groups (166-173 ppm), benzaldehyde (191-192 ppm) and cinnamaldehyde (193-194 ppm) groups, and carbonyl groups in the composition of quinone structures (181-187 ppm). The resonances at 13.7 and 15.0 ppm are assigned to the methyl carbons in ethylated phenolic and aliphatic hydroxyl moieties respectively (*15*). The sharp resonance at 28.7 ppm is tentatively assigned to the methyl carbon in phenylacetone type structures (according to the ^{1}H-^{13}C correlation 1.4 - 28.7 ppm in HETCOR spectrum). The resonances at 52-54 ppm in ^{13}C NMR spectrum of DL, assigned to the $C\beta$ in pino/syringoresinol and phenylcoumarane structures, decreases substantially in the spectrum of EOL indicating the high reactivity of these structures in the catalytic oxidation (Fig. 7). The total amount of β-O-4 type structures, estimated based on $C\gamma$ resonances in the corresponding structural units at 59.7-62 ppm, decreased from 0.58/C6 in DL to about 0.49/C6 in EOL. In addition, the proportion of β-O-4 structural units containing $\alpha C=O$ increases substantially (Fig. 7). The last fact follows from the strong resonances in EOL ^{13}C NMR spectrum at 62.1 ppm, 106.8 ppm (^{1}H-^{13}C signals correlation 7.2 ppm - 104.8 ppm in HETCOR spectrum) and at 156-157 ppm assigned to $C\gamma$, C-2, 6 and C-4 in β-O-4 units with $\alpha C=O$, respectively. The ^{13}C NMR spectrum of EOL revealed probable increase of biphenyl structures as compared with DL. This conclusion was made based on following features in the EOL spectrum (Fig. 7): (i) substantial decrease of resonance intensity at 137.0-137.9 ppm (C-4 in etherified G, S units) with

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simultaneously increase of signals at 141-144 ppm (C-4 in etherified/non-etherified biphenyl structures); (ii) significant diminishing of signals at 147.5-149.2 ppm (C-3 in etherified/non-etherified G units and C-3, 5 in non-etherified S units) and the appearance of a large shoulder at 153-154 ppm (C-3 in 5-5′biphenyl structures) thus broadening the peak at 152.2 ppm (C-3,5 in etherified S units); (iii) appearance of a notable resonance at 128.5 ppm (C-5 in 5-5′ biphenyl structures). The formation of biphenyl type structures during the catalytic oxygen delignification is a predictable oxidation pathway (Eq. 8) and effects negatively the delignification.

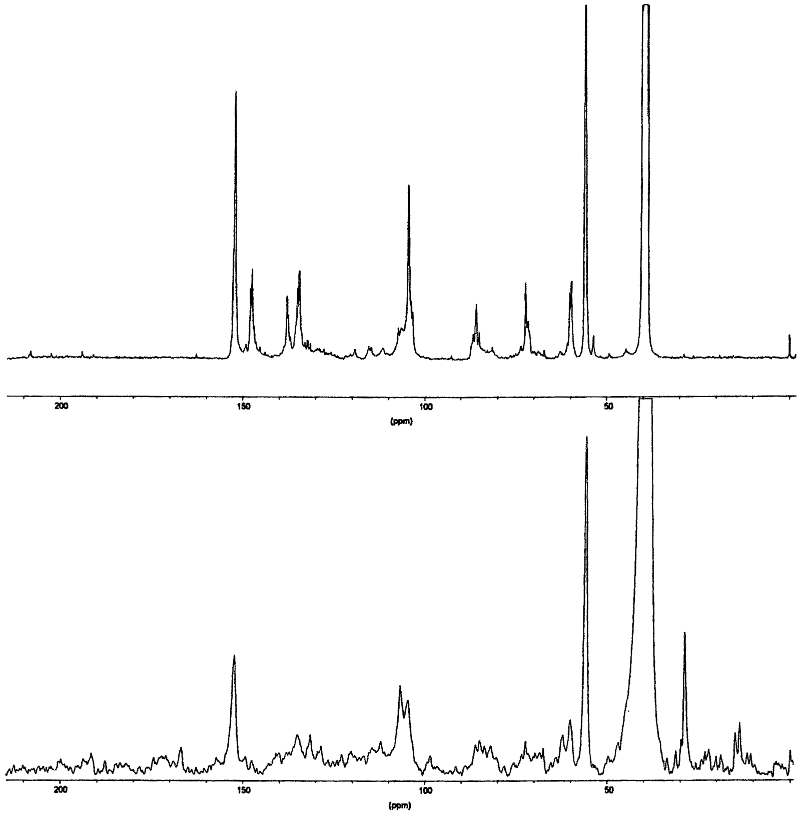

Figure 7. ^{13}C NMR spectra (DMSO-D_6) of eucalyptus dioxane lignin (top image) the lignin isolated from the pulping liquor (bottom image). Acquisition conditions were the same as described in work (15).

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Unlike the oxidation of β-O-4 structures in lignin, the reaction of β-aryl ether dimeric lignin model compounds with HPA-5 is not accomplished by the oxidation of benzylic carbon to αC=O (see Figs. 4, 5). This fact may be explained, at least partially, by problems of the catalyst diffusion in the solid matrix of wood. Figure 8 demonstrates the obvious difficulties of HPA-n ($n < 5$) to reach all the lignin reaction centers. In this context the VO_2^+ ions dissociated from the HPA-5 structure (Eq. 5) can be considered as probable oxidants.

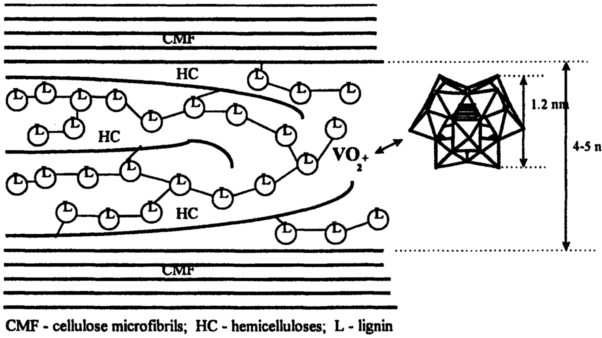
CMF
HC
L
HC
HC
VO_2^+
1.2 nm
4-5 nm
CMF
CMF - cellulose microfibrils; HC - hemicelluloses; L - lignin

Figure 8. The penetration of HPA-5 and dissociated VO_2^+ ions to lignin reaction sites in the lignified cell wall (schematic representation).

This issue was previously discussed based on delignification kinetic studies (*4*). VO_2^+ ions being one-electron oxidants should oxidize the phenolic lignin structures in the manner different than that in homogeneous oxidation in the presence of HPA-n ($1 < n < 5$), which are considered as multi-electron oxidants. Previously, the conversion of a phenolic β-aryl ether lignin model compound containing benzyl alcohol group to the correspondent α-ketone structure was suggested in oxidation with $[SiW_{11}V_1O_{40}]^{5-}$, which is an one-electron oxidant (*1*). Thus, the catalyst diffusion problems in the delignification with HPA-5 influence the lignin oxidation mechanisms.

Conclusions

The studies on lignin oxidation behavior in reaction system HPA-5/O_2 revealed diverse mechanisms involved in the delignification. The rate-limiting step is oxidation of lignin with catalyst (both by HPA-n and VO_2^+ dissociated from the parent heteropolyanion) followed by cleavage of lignin bonds through heterolytic and homolytic mechanisms. The phenolic lignin structural units are those mostly involved in the oxidation. The acidolytic and autooxidative stages

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taking place contribute to the delignification in much lower degree than the oxidation with catalyst species. The autooxidation pathways are not completely disclosed yet. The radical coupling and acid-catalyzed condensation inserting a negative influence on delignification are especially important for the guaiacyl type lignin. The heterogeneous character of HPA-5 catalysis in oxidative delignification influences the lignin reaction mechanisms. In this context the results of mechanistic studies with lignin model compounds in homogeneous oxidation should be taken with a certain care.

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Chapter 21

Catalytic Oxidative Delignification with Keggin-Type Molybdovanadophosphate Heteropolyanions

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Heptamolybdopentavanadophosphate heteropolyanions of the type $[PMo_{(12-n)}V_nO_{40}]^{(3+n)-}$, with 1<n<6, were investigated as regenerable catalysts in the oxidative delignification of wood and pulps by O_2 and O_3. The heteropolyanion $[PMo_7V_5O_{40}]^{8-}$ (HPA-5) was concluded to be the most promising catalyst. The delignification results obtained clearly show the potential of HPA-5 as a lignin oxidation promoter. The use of HPA-5 in oxygen and ozone bleaching processes increases the extent and the selectivity of delignification. The possibility of integrating O_2 and O_3 catalyzed stages in ECF and TCF bleaching sequences is demonstrated, opening new perspectives for the implementation of the closed-mill concept and for the reduction of the environmental impact of bleached kraft pulp mills.

In the last two decades, great attention has been devoted to oxygen and ozone as environmentally friendly delignification/bleaching agents, alternative to chlorine based chemicals (*1*). However, the progressive application of new bleaching technologies has been seriously hindered by the low selectivity of lignin oxidation in these processes. The radical mechanisms involved in such systems are hard to control and lead to the formation of hydroxy and

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ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

hydroperoxy radicals in lignin autooxidation stages. These radicals are non-selective oxidants which attack polysaccharides, decreasing pulp properties and, for this reason, restrain the achievement of high degree of delignification.

One possibility to overcome the drawbacks of such processes may be the use of selective catalysts that control the reactivity of oxygen or other oxygenated chemicals thus avoiding lignin autooxidation and increasing the selectivity of delignification. Promising results have been obtained with catalysts such as transition metal complexes and metalloporphyrins that mimic the active centers of enzymes responsible for the lignin biodegradation in nature (ligninases) (*2-4*). However, the practical application of these catalysts is limited by their high cost, instability towards oxidation and regeneration problems. Recently, inorganic metal-oxygen cluster anions (polyoxometalates), a family of catalysts known by their efficiency in oxidative organic synthesis (*5*), were proposed in new oxidative delignification/bleaching approaches (*6, 7*). Easily synthesized and not too costly, polyoxometalates (POMs) are stable under a wide temperature range and can be re-oxidized and re-used in the oxidation processes.

POMs have been proposed as regenerable stoichiometric oxidants for pulp bleaching (*6, 9-11*). According to this approach, the pulp is bleached with POM under anaerobic conditions, followed by an alkaline extraction stage to remove oxidized residual lignin. The regeneration (re-oxidation) of reduced POM species in the bleaching filtrate is carried out by oxygen in a separate stage, using temperatures higher than those applied in the bleaching stage. This multistage bleaching approach requires high concentrations of POM (0.05-0.1 mol/l) and long treatment times to achieve good delignification .

In 1996, our research group proposed the use of molybdovanadophosphate heteropolyanions of the series $[PMo_{(12-n)}V_nO_{40}]^{(3+n)-}$ (HPA-n with $1<n<6$), POMs with Keggin-type structure, as selective catalysts in the aerobic oxidative delignification of lignocellulosics, including wood and pulps (*7, 12-23*). The molybdovanadophosphate heteropolyanion $[PMo_7 V_5O_{40}]^{8-}$ (HPA-5) was shown to be perfectly adapted for the catalytic purposes. In the aerobic process the lignin is selectively oxidized by HPA-5, under oxygen atmosphere. After reaction with lignin, the reduced HPA-n is immediately re-oxidized by oxygen in same process stage. The redox cycle is continuously repeated. This single-stage process is carried out in the presence of low HPA-5 concentrations (1-3 mmol/l). A high degree of delignification of hardwood kraft pulp can be achieved in 1.5-2.0 h of treatment. Following the promising results with HPA-5 catalyzed oxygen delignification, the same catalytic approach was extended to oxidative delignification with ozone.

The fundamentals and mechanisms of this new catalytic delignification approach have been summarized in the previous chapter of this book (*24*). The present chapter summarizes the main results of HPA-n catalyzed delignification

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of wood and pulps with oxygen, and ozone, including the application of HPA-5 catalyzed stages in ECF and TCF bleaching sequences.

Principles of HPA-5 Catalyzed Delignification

In POM-based oxidative delignification systems, lignin is oxidized with POM, followed by the re-oxidation of the reduced POM, by a suitable oxidant such as O_2 or O_3 according to the scheme:

$\text{Lignin} + \text{POM}_{ox} \rightarrow \text{Lignin*} + \text{POM}_{red}$ (1)

$\text{POM}_{red} + \text{Ox} \rightarrow \text{POM}_{ox} + H_2O$ (2)

where POM_{ox} and POM_{red} are the oxidized and reduced forms of POMs respectively, Lignin* is the oxidized form of the lignin and Ox is the oxidant. In the case of HPA-n, the oxidation occurs due the ability of V(V) to accept electrons from lignin. While in the anaerobic approach (POM used as stoichiometric oxidants) the two reactions occur in different process stages, in the catalytic (aerobic) approach, reactions (1) and (2) occur simultaneously in the same stage. To achieve this catalytic cycle, continuously repeated, the thermodynamic conditions must be as follows:

$E^o(\text{Lignin}) < E^o(\text{POM}) < E^o(O_2, \text{ or } O_3)$

Thus, the catalyst should have a redox potential high enough to oxidize the lignin and, at the same time, lower than that of the oxidant. The potential of oxidation peak of different lignin phenolic units are in the range +0.45 to +0.69 V (vs NHE, pH 2) (*23*), while redox potentials of O_2 and O_3 are +1.23 and +2.07 V (vs NHE, pH 1.0), respectively (*25*). The E_p of HPA-5 is estimated to be about + 0.60 V (vs NHE, pH 2) (*23*).

HPA-n are normally stable at pH 3.0-6.0. However, at pH < 3, HPA-n undergo partial dissociation:

$\text{HPA-n} \leftrightarrow \text{HPA-(n-x)} + xVO_2^+$ (3)

The VO_2^+ ions produced via HPA-n dissociation (Eq.(3)) have an E_p of about 0.90 V vs NHE at pH 2 (*23*), a figure higher than the E_p of the parent HPA-n. Thus, in the system HPA-5/O_2, the thermodynamic conditions required to achieve the cyclic catalytic delignification are fulfilled. Conditions favoring the partial dissociation of HPA-5 (leading to the formation of HPA-n, with n<5, and VO_2^+ ions) increase the oxidation capacity of the catalytic solution. (12). For this reason, HPA-5 catalyzed delignification experiments are carried out at the pH < 2.5 (*13,16*). Apart from the positive role in delignification, VO_2^+ ions have an undesirable effect on the oxidative degradation of polysaccharides. The extent of VO_2^+ dissociation from the parent HPA-n can be regulated through pH control, addition of polar organic solvents or by increasing the ionic strength of the solution (16-20).

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The selective oxidation of lignin in wood or pulp is possible due to the higher electron-donor character of lignin (compared to the polysaccharides), and to the suppression of radical chain reactions by rapid reduction of oxy-radicals by V(IV) in VO^{2+} or in HPA-5 composition.

HPA-5 Catalyzed Oxygen Delignification of Wood

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The effect of HPA-5 was initially tested in the oxygen delignification of eucalyptus sawdust in an ethanol-water medium and in aqueous solutions (*12,13*). The results are summarized in Table I. The addition of 2 mM HPA-5 to the ethanol-water pulping solution increases substantially the extent of delignification when compared with control experiments without HPA-5 or in the presence of 0.01 M H_2SO_4 (used to simulate the acidic conditions used in the HPA-5 catalyzed experiments). In the presence of HPA-5 (2mM, pH 1.8), 76.5 % of wood delignification is achieved at temperatures as low as 100 °C, against only 15 % or 24% in the absence of HPA-5, without or with addition of sulfuric acid, respectively. The partially reduced form of HPA-5 is slightly more effective in delignification than the oxidized form. This effect was assigned to the increased extent of HPA-5 dissociation and liberation of VO_2^+, when partially reduced (*13,16*). When the catalytic delignification is carried out in an aqueous medium, the degree of delignification and pulp yield are lower than in an ethanol-water medium due to stronger lignin condensation and polysaccharides degradation (*20,22*). In the following studies, the reduced form of HPA-5 was used.

The pH of the pulping solution strongly influences HPA-5 catalyzed oxygen delignification. Polysaccharides dissolution increases with decreasing pH. On the other hand, the degree of delignification remains approximately constant between pH 1.2 and pH 1.8, decreasing markedly at higher pH. Therefore, the optimal pH is in the range 1.8-2.0. The decrease of delignification efficiency at pH higher than 2 is explained by an increase in HPA-5 stability and, thus, to a decrease of free VO_2^+ ions in solution (*13,16*).

The concentration of HPA-5 also shows a pronounced effect on delignification. Maximum delignification was registered at nearly 2 mM concentration. The decrease in the oxidizing ability at concentrations above 2-3 mM (at constant pH) is probably a consequence of the increase in ionic strength in ethanol-water solutions, due to addition of NaOH for pH adjustment. The spruce sawdust delignification occurred in a manner similar to that of eucalyptus sawdust (*13*).

Table I. HPA-5 Catalyzed Oxygen Delignification of Wood

Samples	*Pulping solution*	*Pulp Yield, %*	*Degree of Delignification, %*
Wood sawdust	EtOH-H_2O, without HPA-5	95.6	14.8
	EtOH-H_2O, H_2SO_4 0.01 M	87.9	23.8
	EtOH-H_2O, [HPA-5_{red}]=2 mM, pH=1.2	59.9	78.4
	EtOH-H_2O, [HPA-5_{red}]=2 mM, pH=1.8	68.5	76.5
	EtOH-H_2O, [HPA-5_{red}]=2 mM, pH=2.8	91.9	26.1
	EtOH-H_2O, [HPA-5_{red}]=0.3 mM, pH=1.8	81.8	48.5
	EtOH-H_2O, [HPA-5_{red}]=8 mM, pH=1.8	85.2	32.4
	EtOH-H_2O, [HPA-5_{ox}]=2 mM, pH=1.8	73.3	67.2
	H_2O [HPA-5_{red}]= 2mM, pH=1.8	62.8	67.2
Wood chips	EtOH-H_2O (50:50, v/v), [HPA-5_{red}]=2mM	62.2*	81.5*

NOTES: T=100°C in eucalyptus sawdust experiments, 110 °C in eucalyptus chips experiment; t=5 h; liquor-to-wood ratio=5; pH=1.8 (for pulping solutions containing HPA-5), p^0O_2=0.7 MPa; ethanol-water solution composition: 50:50 (v/v). (*) In wood chips delignification, yield figure refers to 51.0 % pulp + 11.2 % uncooked chips; the degree of delignification figure refers to pulp (not including uncooked chips)

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The HPA-5 catalyzed oxygen delignification was also tested in the delignification of wood chips. However, results were not very encouraging. Delignification was always limited to the external regions of the chips, because of the hard diffusion of catalyst into wood tissue (*14*). The problem may be partially overcome by increasing the temperature, but, in this situation, the selectivity of delignification is strongly decreased.

The analysis of pulping liquors showed that lignin and dissolved organic matter are almost completely converted to CO_2 and H_2O and that the catalyst remains stable after the delignification process (*13*). These features, together with the highly selective delignification obtained in wood sawdust, stimulated us to extrapolate the HPA-5 catalytic delignification approach to the bleaching of chemical pulps.

HPA-5 Catalyzed Oxygen Bleaching of Kraft Pulp

The oxygen bleaching experiments in the presence of HPA-5 (*13, 15, 17-19*) were carried out in ethanol-water media and in aqueous media (Table II).

Table II. HPA-5 Catalyzed Oxygen Bleaching of Kraft Pulp and its Comparison with Conventional Alkaline Oxygen Bleaching.

Bleaching conditions	*Pulp Yield, %*	*Kappa number*	*Bright-ness, %*	*[η], cm^3/g*
Initial Kraft pulp	100	16.9	23	1270
EtOH-H_2O (50:50 v/v) [HPA-5_{red}]=2mM 90°C, pH 1.9, 120 min.	96.3	6.6	62	975
H_2O, [HPA-5_{red}]=2mM 90°C, pH 1.9, 120 min.	93.2	5.2	-	620
H_2O, NaOH (2% on pulp) 90°C, 120 min.	95.8	10.4	43	1100
H_2O, NaOH (2% on pulp) 90°C, 160 min.	94.2	9.1	-	885
H_2O, NaOH (2% on pulp) 106°C, 80 min.	93.8	6.3	61	755

NOTES: Consistency of eucalyptus kraft pulp was 3%, P^0O_2=0.7 MPa

Under the conditions used, delignification degrees of the order of 60-70% were obtained , with viscosity and pulp yield losses of the order of 20-50% and 4-7%, respectively. The nature of the delignification medium strongly influences the bleaching process. In aqueous media, the degree of delignification is higher but the viscosity is lower than in ethanol-water media.

In HPA-5/O_2 bleaching, the degree of delignification is generally twice that obtained for oxygen bleaching in alkaline medium, under similar treatment conditions. In order to achieve comparable results, the alkaline process requires more drastic conditions than the HPA-5-based process. However, under such conditions, polysaccharides are readily oxidized and pulp viscosity decreases significantly. The degradation of cellulose under HPA-5/O_2 bleaching was found to be determined essentially by acid catalyzed hydrolysis, but also by oxidation reactions (*20*). Oxidative depolymerization is associated mainly with the action of VO_2^+ ions produced via partial dissociation of HPA-5 under acidic conditions.

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The effect of the composition of the ethanol-water media was investigated. When ethanol is added to the bleaching mixture (Fig. 1) in 0-20% vol. concentration, the kappa number and viscosity of the pulp increases. For 20-50% ethanol concentrations, the increase in kappa number is not significant while the viscosity continues to increase. For ethanol concentrations higher than 50 %, the degree of delignification decreases dramatically. The addition of ethanol to HPA-5 solution increases its stability and hinders the formation VO_2^+. Thus, according to the results shown in Fig. 1, an increase in the concentration of VO_2^+ ions (decrease of ethanol concentration) leads to higher delignification rates but also to lower delignification selectivity. This suggests that VO_2^+ ions are stronger oxidizing agents than the parent HPA-5 but also less selective for lignin oxidation. In this way, ethanol in solution regulates the oxidation capacity and selectivity of the reaction system.

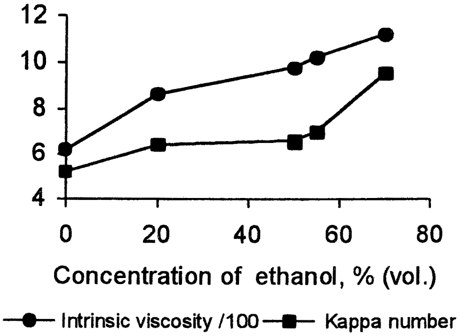

Figure 1. Effect of the composition of the reaction medium on the bleaching in HPA-5/O_2 system ([HPA-5]=2mM, T=90°C, $P^{o}O_2$=0.5 MPa, pH=1.8, t=2h, consistency=3%)

The best temperature for the bleaching was found to be in the range 85-90 °C. At higher temperatures, although delignification still increases, there is a dramatic drop in pulp viscosity. Delignification was found to be independent of pulp consistency in the range 3-10 %. Hence, variations in the pulp/catalyst ratio do not affect the final bleaching results, suggesting that the catalyst regeneration stage is not the limiting step in oxidative delignification. Delignification was found to be independent of the oxygen pressure. The only requirement is to have enough oxygen to re-oxidize HPA-5. O_2 pressure as low as 0.2 MPa may be used. However, because, our experiments were carried out in a closed batch reactor, an excess of oxygen was always used (0.5-0.7 MPa) to avoid O_2 depletion in the reactor.

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Although it has been previously shown that the catalyst remains stable after a delignification experiment, it was necessary to check the possibility of using the same catalyst solution in multiple bleaching experiments. A series of ten bleaching experiments was carried out using as bleaching solutions, the filtrate from the previous bleaching experiment. Results from pulp delignification revealed no significant changes in kappa number and viscosity after the ten bleaching cycles, showing that no deactivation occurred during the experiments performed (Fig. 2) (*15*). The accumulation of oxidized and dissolved organic substances in filtrates was followed by determination of COD index. After the first two cycles, no changes in COD were observed (Fig. 2). Thus, no further accumulation of organic matter occurred with the consecutive use of the same liquor in pulp bleaching. This is achieved because of the conversion of lignin and other organic material to CO_2. Therefore, the HPA-5/O_2 bleaching can be carried out in a closed system since the bleaching liquor may be continuously re-used.

Although bleaching results obtained in ethanol-water media are better than in aqueous media, the use of organic solvents is not very attractive from the point of view of eventual industrial applications. A possible solution is to decrease the extent of dissociation of HPA-5 in aqueous media (decreasing the formation of VO_2^+) by increasing the ionic strength of the aqueous bleaching solution. The increase in ionic strength should be also beneficial from the point of view of cellulose hydrolysis, since proton activity is reduced. Sodium sulfate was added to the aqueous bleaching solutions as way of increasing the ionic strength (*18*). The addition of sodium sulfate leads to an increase in the pulp viscosity and decrease in delignification (Fig. 3). With sodium sulfate concentrations of 0.1-0.2 mol/l, delignification degrees and viscosity of pulps obtained in aqueous media are similar to those obtained in ethanol/water systems. Thus, the need to use an organic solvent is overcome by the addition of sodium sulfate to the aqueous media.

A comparison of the HPA-5/O_2 delignification of softwood and hardwood (eucalyptus) kraft pulps showed the lignin removal rates about two times slower for the softwood pulp (26). This fact was explained by the residual lignin in the softwood, which is more condensed and more difficult to oxidize with HPA-5 than that in the eucalyptus pulp. In addition, during the oxidative treatment at low pH (90°C; pH 2; 2h; aqueous solution (2 mmol/l) of HPA-5 in the presence of Na_2SO_4 (0.1 M)), the residual lignin of softwood pulp suffered a notable condensation. All these features hindered the delignification of softwood pulp in the O_{HPA-5} bleaching stage (26). However, after alkaline extraction ($O_{HPA-5}E$ sequence), due to the high solubility of oxidized lignin, the delignification degrees achieved for both softwood and hardwood pulps were similar (about 60 %) (26).

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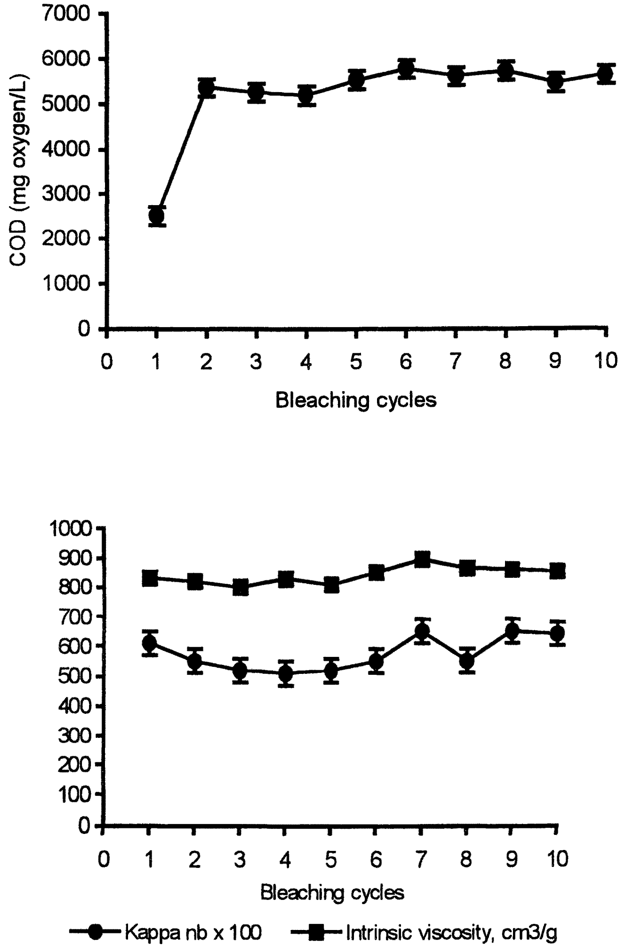

Figure 2. Influence of the multiple use of same HPA-5 solution on kappa number and intrinsic viscosity of pulps and COD of bleaching filtrate (EtOH-H_2O 50:50, [HPA-5]=2mM, T=90°C, p^0O_2=0.5 MPa, pH=1.8, t=2h, consistency=3%)

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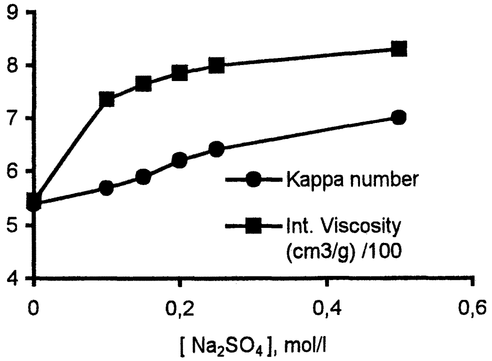

Figure 3. Effect of the addition of Na_2SO_4 in the aqueous HPA-5/O_2 bleaching system ([HPA-5]=2mM, T=90°C, P^0O_2=0.5 MPa, pH=1.8, t=2h, consistency=3%).

HPA-5 Catalyzed Ozone Bleaching of Kraft Pulp

The ozone bleaching experiments (*18,19*) were carried out in aqueous solutions at pH 2, using different HPA-5 concentrations (Table III). Results clearly show a significant catalytic effect of HPA-5 on ozone pulp bleaching. At the lowest applied catalyst concentration (0.4 mM) the best results for delignification (nearly 50 %) and pulp brightness were obtained, as compared with control experiment without the catalyst. However, a decrease in bleached pulp viscosity takes place (Table III). The increase in the catalyst concentration leads to a decrease in the delignification rate and an increase in pulp viscosity. At an HPA-5 concentration of 1.8 mM, better delignification and pulp viscosity are obtained when compared to those for pulp in the control bleaching experiment without catalyst (Table III). Thus, in addition to the catalytic effect, HPA-5 seems to protect the pulp polysaccharides against oxidative degradation. This proposition was confirmed by the analysis of oxidized groups in HPA-5/O_3 bleached pulps, which showed a ketone group content three times lower than that of the pulp bleached without catalyst (*18*). The protective effect of HPA-5 was suggested to be associated with the reduction of hydroxyl radicals (formed by ozone decomposition or lignin autooxidation reactions) by V(IV) in HPA-5 composition or as free VO^{2+} ions.

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Table III. HPA-5 Catalyzed Ozone Bleaching of Eucalyptus Kraft Pulp in Aqueous Media.

Bleaching conditions	*Kappa number*	*$[\eta]$, cm^3/g*	*Brightness, %*
Initial eucalyptus kraft pulp	12.8	1130	41.5
No catalyst	8.4	995	49.5
[HPA-5]=0.4mM	6.3	930	50.5
[HPA-5]=0.9mM	7.8	955	50.0
[HPA-5]=1.8mM	7.8	1030	48.0

NOTES: T=25°C, pH=2, t=20min, pulp consistency=5%, ozone rate=42mg/min)

Bleaching Sequences Including HPA-5 Catalyzed Stages

Aqueous and ethanol-water HPA-5/O_2 delignification was tested in ECF bleaching sequences (*15,17*), as a pre-bleaching stage (Table IV). The $O_{HPA\text{-}5}EDD$ sequences allow a 50-65 % reduction in ClO_2 consumption (~20 Kg ClO_2 per ton of pulp), when compared with standard DEDED sequences, with a decrease of 10-15% in strength parameters. The ozone and/or oxygen catalyzed bleaching in aqueous media was also investigated in TCF bleaching sequences (Table IV) (*19*): $O_{HPA\text{-}5}E_PPPP$, $O_{HPA\text{-}5}Z_{HPA}E_PP$ and $QZ_{HPA\text{-}5}Z_{HPA\text{-}5}E_PPP$.

In the $O_{HPA\text{-}5}E_PPPP$ sequence, the oxygen pre-bleaching stage gives about 50 % delignification (expressed as kappa number) while reducing pulp viscosity by 25 %. About 20-25 % of the kappa number reduction in the $O_{HPA\text{-}5}$ stage is achieved due to the elimination of hexenuronic acid residues (HexA) normally present in the kraft pulp (*19*). Further peroxide treatment leads to a pulp with 84 % brightness. The implementation of the ozone stage just after the oxygen delignification ($O_{HPA\text{-}5}Z_{HPA}E_PPP$) leads to an increase of the pulp delignification, before the peroxide bleaching, up to about 60 % without substantial loss of pulp viscosity. A quite similar degree of delignification (~55 %) is achieved using only HPA-5 catalyzed ozone stages ($QZ_{HPA\text{-}5}Z_{HPA\text{-}5}E_PPP$) before bleaching with peroxide. However, in this situation, the viscosity drop is much lower than in the previous sequences, a consequence of the protection of polysaccharides by HPA-5 during ozone bleaching. Ozone catalyzed stages in the $QZ_{HPA\text{-}5}Z_{HPA\text{-}5}E_PPP$ sequence leads to a higher kappa number reduction and a smaller decrease in pulp viscosity than in sequence with non-catalyzed ozone delignification $QZZE_PPP$. The viscosity of the $QZ_{HPA\text{-}5}Z_{HPA\text{-}5}E_PPP$ pulp is only 5% lower than that of the conventional ECF (DEDED) pulp.

Hence, the use of O_2 and/or O_3 catalyzed stages in TCF sequences gives pulps with 83-84 % brightness with low hydrogen peroxide charges (20-30 Kg of H_2O_2) in the following stages. The pulps obtained have physical properties better than those obtained from non-catalyzed TCF bleaching sequences and only slightly lower than those obtained by ECF (DEDED) sequence (Table V).

Conclusions

HPA-5 catalyzed bleaching is a promising approach for new environmentally friendly bleaching techniques based on oxygen and ozone. HPA-5 increases the extent of delignification and reduces the degradation of polysaccharides. Pulps with properties equivalent to those obtained by conventional bleaching sequences, or even better in the case of TCF, may be obtained using O_2 and O_3 catalyzed stages. During the bleaching procedure, HPA-5 is continuously regenerated and the bleaching filtrate can be re-used. In

Table IV. Results of Kraft Pulp Bleaching with TCF Sequence Including HPA-5 Catalyzed Stages in Aqueous Media. Comparison with Conventional ECF Sequence.

Bleaching sequences	*Bleaching stage*	*Kappa number*	*Brightness, %*	*[η], cm^3/g*
Initial kraft pulp		13.8	38	1160
$O_{HPA-5}E_pPPP$	O_{HPA-5}	7.5	56	860
	E_pPPP	-	84	765
$O_{HPA-5}Z_{HPA-5}E_pPP$	O_{HPA-5}	7.5	56	860
	Z_{HPA-5}	5.8	66	835
	E_pPP	-	84	785
$QZ_{HPA-5}Z_{HPA-5}E_pPP$	QZ_{HPA-5}	8.9	58	1125
	Z_{HPA-5}	6.7	64	1080
	E_pPP	-	83	855
$QZZE_pPP$	QZZ	7.2	-	1015
	E_pPP	-	82	775
DEDED		-	88	945

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Table V. Strength Properties of Pulps bleached by TCF Sequences Including HPA-5 Catalyzed Stages. Comparison with Non-Catalyzed TCF Sequence and Conventional ECF Sequence.

	$O_{HPA-5}E_pPPP$	$QZ_{HPA-5}Z_{HPA-5}E_pPP$	$QZZE_pPP$	DEDED
PFI rotations	1000	1000	750	2000
°SR	31	37	44	32
Tensile, N.m/g	68.2	75.2	69.1	77.9
Tear, mN.m^2/g	8.32	8.49	4.08	9.01
Burst, KPa.m2/g	5.27	6.01	4.59	5.35
Lw, mm	0.72	0.73	0.68	0.73

this way HPA-5 bleaching opens new perspectives for the implementation of the closed-mill concept and for the reduction of the environmental impact of bleached kraft pulp mills. However, the HPA-5 catalyzed process should be further improved. Because of the low pH used, some hydrolytic degradation of cellulose takes place. Work is in progress to search for new heteropolyanions bearing catalytic activity in reaction media with pH ≥ 3.

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Chapter 22

Characterization of Residual Lignins in Pulps Delignified by Laccase/*N*-Hydroxyacetanilide

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A pine kraft pulp and a two-stage oxygen-delignified pine kraft pulp were delignified with laccase in the presence of N-hydroxyacetanilide (NHA). Laccase/NHA was selective in its reactions with lignin: 30% delignification was obtained under the moderate reaction conditions employed, while the carbohydrates remained unattacked. Laccase/NHA did not react with neutral pulp carbohydrates or with uronic acids. Beside delignification, laccase/NHA caused chemical modification of fiber lignin, lowering the content of free phenolic hydroxyl groups and thus verifying their importance in delignification. Oxidation of the residual lignins was clearly observed as a decrease in methoxyl groups and a simultaneous increase in conjugated carbonyl groups, carboxyl and/or ester groups. Only 50-60% of the pulp residual lignin retained its aromatic structure. Pyrolysis-GC/MS gave important information, not only of the chemical structure of residual lignins, but also of the behavior of the mediator during delignification. Residuals of aniline and acetanilide, i.e. degradation products of NHA, were found among the pyrolysis products of residual lignins. However, only trace amounts of NHA-derived nitrogen-containing products were present in the lignins.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Introduction

Laccases are lignin oxidizing enzymes, whose potential for pulp bleaching has been widely investigated since the invention of the laccase/mediator system, which enables the large enzyme molecule to react with fiber lignin via the small molecular mediator molecule (*1*). Investigations in this area are now aimed primarily at finding new efficient mediators with low production costs. Suitable mediators have been found to be compounds containing the N-OH group. One of the most widely studied mediators is 1-hydroxybenzotriazole (HBT) (*1*). More recently developed mediators include violuric acid and N-hydroxyacetanilide, NHA (*2*), which was chosen for the present study. It has been suggested that NHA is superior to HBT as a mediator, because, unlike HBT, it does not inhibit laccase and is a more specific laccase substrate. It is also biodegradable and contains less nitrogen per molecule than HBT. NHA is also cheaper to produce than HBT (*2*).

Pulps delignified with laccase in the presence of mediator are known to be more reactive towards final bleaching than the reference pulps *(1,2,3,4,5)*. In order to explain this increased reactivity, as well as the reactions occurring during delignification, the chemical structures of the pulps themselves and of the residual lignins from a pine kraft pulp and from an oxygen-delignified pine kraft pulp before and after laccase/NHA delignification were studied. NHA was also compared to HBT as a mediator in laccase delignification.

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Materials and Methods

Pulps and laccase.

Pine (*Pinus sylvestris*) kraft pulp was prepared in the laboratory. The sulphidity was 35% and the effective alkalinity was 3.9 mol/kg. The pulp was subjected to a two-stage oxygen delignification (OO). The oxygen stages were carried out at 95°C for 60 min and 75 min using 2.0% and 1.4% NaOH, respectively, and at 0.8 MPa oxygen pressure at 10% consistency. The $MgSO_4x7H_2O_2$ charge was 0.5% on pulp in both stages. The properties of the pulps are shown in Table I.

Laccase was produced by *Trametes versicolor* and was kindly donated by Consortium für electrochemische Industrie GmbH.

Pulp Treatments

Laccase/NHA Treatment (L/NHA). Never-dried pulp (60 g abs. dry) was treated with laccase (15 IU/g pulp = 250 nkat/g pulp) in the presence of NHA (obtained from Consortium für electrochemische Industrie GmbH) at pH 4.5 and 45°C for 2 hours under 0.3 MPa oxygen pressure at 10% consistency in a rotating autoclave. The NHA charge was 1.83% (121 mmol/kg pulp) on pine kraft pulp and 0.73% (48 mmol/kg pulp) on oxygen-delignified pulp. Thus the ratio of NHA to lignin was the same in both pulps. Oxygen-delignified pulp was also treated with laccase in the presence of 1.12% NHA (74 mmol/kg pulp).

Laccase/HBT treatments were performed under the same conditions with 1.00% HBT (74 mmol/kg pulp) on oxygen-delignified pulp.

Laccase treatments (L) without NHA and reference treatments without laccase or NHA were carried out under the same conditions as described for the laccase/NHA system.

Alkaline extraction (E) was carried out at 10% pulp consistency, with 1% sodium hydroxide on pulp at 60°C for 1 h with occasional stirring.

Residual lignins

Residual lignins were isolated from the pine kraft pulp (NHA: 1.83%) and from the OO-delignified pine kraft pulp (NHA: 0.73%) before and after laccase and laccase/NHA treatments using an enzymatic method (*6*). The lignins were further purified as described earlier (*7*).

Analyses

Kappa number and *viscosity* were determined using the standard methods SCAN-C 1:77 and SCAN-CM 15:88, respectively. Pulp brightness was measured with an Elrepho instrument. *Polysaccharides* were hydrolysed to monosaccharides, which were quantitatively determined by anion exchange chromatography (*8*). The pulps were subjected to total enzymatic hydrolysis and the *4-O-methylglucuronic acid* (MeGlcA) and *hexenuronic acid* (HexA) contents were determined from the hydrolysates (9). *Lignin kappa number* was calculated using the reported finding that 10 mmol HexA/kg pulp is equal to 0.86 kappa units (*10*).

Carbonyl group and *carboxyl group* contents of the pulps were determined according to the published methods (*11,12*).

Pyrolysis-GC/MS of the samples was based on an earlier work (*13*). The pulps and residual lignins were pyrolysed at 580°C for 2 s (Pyrolab 2000), and the resulting fragments separated and identified using GC/MS (Varian 3800 and Varian Saturn 2000).

Nitrogen analysis and methoxyl group determinations of the lignins were performed at the Analytical Laboratories, Engelskirchen, Germany. The protein content was obtained by multiplying the percentage nitrogen content by 6.25 (*14*).

Phenol groups in the residual lignins were determined using an ionization difference-UV method (*14*).

FTIR spectra were obtained with a Nicolet 740 FTIR spectrometer (KBr technique). FTIR spectra corrected for the purified residual lignins were

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obtained by subtracting the FTIR spectrum of the protein impurity from the FTIR spectra of residual lignins (*14*).

Results and Discussion

Delignification by Laccase/NHA

A pine kraft pulp and the same pine kraft pulp after a two-stage oxygen delignification were treated with either laccase alone or laccase in the presence of NHA as a mediator. The ratio of mediator to lignin was the same for both pulps. Table I shows the properties of the pulps before and after the treatments. The kappa numbers of the pine kraft pulp and the oxygen-delignified pulp were lowered by 27% and 25%, respectively, by laccase/NHA and subsequent alkaline extraction under the conditions used. Kappa number reduction by laccase was no higher than that in the reference treatment under the same reaction conditions without laccase. Taking the content of hexenuronic acids into account (lignin kappa number), the delignification achieved with laccase/NHA was calculated to be 29% for the pine kraft pulp and 31% for the oxygen-delignified pulp.

The hexenuronic acid content of pine kraft pulp and oxygen-delignified pine kraft pulp was 23 mmol/kg; this was unaffected by the laccase and laccase/NHA treatments (Table I). The results thus clearly show that laccase in the presence of NHA did not react with the double bond in hexenuronic acid either under oxygen pressure or at atmospheric pressure (*15*). The methylglucuronic acid content of the pulps was insignificant.

The high pulp yields, and the fact that there were no changes in pulp viscosity and no significant changes in total carbohydrate content and composition suggest a high selectivity of laccase/NHA towards lignin (Table I). These benefits have also been reported for laccase/HBT treatments (*3*).

Laccase/NHA treatment increased the content of carboxyl groups and carbonyl groups in pine kraft pulp. As with laccase/HBT treatment (3), both are probably introduced into lignin and not into carbohydrates. The low lignin content of oxygen-delignified pulp after laccase/NHA treatment prevents any increase in carbonyl group content from being seen. Unlike with laccase/HBT delignification *(3),* the carboxyl content decreased. Dissolution of the highly oxidized lignin in the OO-pulp during laccase/NHA treatment can explain the overall drop in the carboxyl content of the pulp in spite of the oxidative conditions during the treatment.

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Table I. Properties of Pine Kraft Pulp and Two-Stage Oxygen-Delignified Pine Kraft Pulp (OO), Before and After Laccase Treatment and Laccase/NHA Treatment and Subsequent Alkaline Extraction (E).

	Kraft	*LE*	*(L/NHA)E*	*OO*	*OOLE*	*OO (L/NHA)E*
Kappa no.	25.4	22.4	18.6	10.1	9.1	7.6
Lignin kappa no.	23.4	20.3	16.7	8.1	7.1	5.6
Brightness, %	30.4	30.1	23.2	38.9	44.3	42.5
Viscosity, ml/g	1110	1140	1170	890	890	890
Yield, % on wood	45.5	45.1	45.3	43.7	43.3	43.3
HexA, mmol/kg	23	24	22	23	23	23
MeGlcA, mol/kg[a]	+	+	+	+	+	+
COOH, mmol/kg	74.3	75.3	92.8	81.3	79.1	71.8
CO, mmol/kg	15	17	28	17	18	18
Tot. monosacch., mg/100mg	95.5	93.3	94.3	95.8	95.7	94.8
Ara, %	0.8	0.8	0.7	0.7	0.7	0.7
Gal, %[b]	+	0.4	0.4	+	+	+
Glc, %	84.1	84.4	84.4	84.8	85.0	84.8
Xyl, %	8.2	7.7	7.7	7.7	7.5	7.8
Man, %	6.9	6.7	6.8	6.8	6.8	6.7

[a] + = below determination limit of 15 mmol/kg.

[b] +=below determination limit of 0.2 mg/100 mg.

Comparison of Laccase/NHA and Laccase/HBT Delignification

There was no difference in kappa reduction between laccase/HBT and laccase/NHA-treated and alkali-extracted oxygen-delignified pine kraft pulps, when equal amounts (74 mmol/kg pulp) of HBT and NHA on molar basis were used (Figure 1). However, the brightness improvement was a little greater for laccase/HBT-treated pulp than for laccase/NHA-treated pulp. Earlier studies on comparison of mediator indicated that the kappa reduction gained with laccase/NHA was slightly greater (*2,16,17*) for softwood kraft pulps of low kappa numbers and slightly lower (*18*) for softwood kraft pulps of higher kappa numbers compared to laccase/HBT. However, a much higher degree of delignification was obtained with the same HBT charge using a higher charge of a different laccase in the case of an oxygen-delignified pine kraft pulp with slightly lower kappa number *(3)*.

Comparing the effects of NHA charges showed that increasing the NHA dosage is unnecessary, because the 25% kappa reduction was achieved with NHA charges of 48 and 74 mmol/kg pulp.

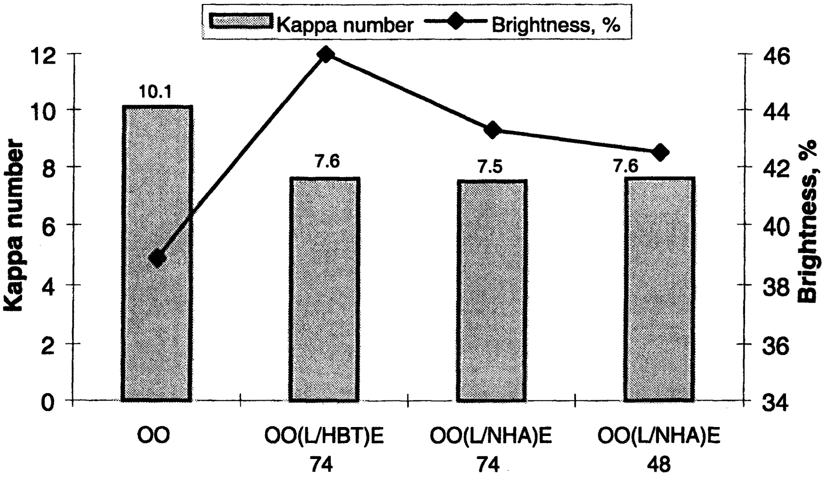

Figure1. Comparison of the effects of HBT and NHA on oxygen-delignified pine kraft pulp, when equal amounts of mediator (74 mmol/kg pulp) were used.

Residual Lignins

The pine kraft pulp and the two-stage oxygen-delignified pine kraft pulp, before and after laccase and laccase/NHA treatment followed by alkaline extraction, were subjected to total enzymatic hydrolysis in order to obtain the residual lignins. The lignin yields obtained ranged from 77% to slightly over 100% calculated from the total lignins in the pulps (Table II). This method (*6*) is known to give the lowest yield for the residual lignin from the pulp with the lowest kappa number, which probably indicates that some hydrophilic part of the lignins was not precipitated.

However, despite the use of protease purification, the isolated lignin still contained some protein impurities originating from the cellulases used in the isolation procedure. The protein contents of the residual lignins were determined from their nitrogen contents (Table II). All nitrogen was assumed to originate from the protein residues of the enzymes. The protein contents of the lignins were also determined by pyrolysis-GC/MS. The protein impurity gives rise to three degradation products, which can be identified and quantified, although the accuracy of this method is not as good as that based on nitrogen content. However, the two methods gave comparable results, indicating that only trace

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amounts of NHA-derived nitrogen-containing products were present in the samples.

Detected as monosaccharides, the total carbohydrate contents of all isolated residual lignins were 6.3-8.2 mg/100mg (Table II), which are also typical values for lignins from other alkaline pulp types, as well as for laccase/HBT-delignified pulps. Some carbohydrates originate from the cellulase enzyme, which contained 10.2% by weight of carbohydrates, the monosaccharide composition being 85% mannose, 8.5% glucose and 6.5% galactose (*14*). Most, however, originated from the carbohydrates in the pulp, representing lignin-carbohydrate complexes. The monosaccharide content and composition of carbohydrates originating from the pulps show that the carbohydrate contents in the residual lignins from the laccase/NHA-treated pulps were slightly lower than in the residual lignin of the initial pulps or of the laccase-treated pulps. The decrease was in the mannose and galactose contents in the case of laccase/NHA-treated pine kraft pulp lignin and in the mannose content in the case of laccase/NHA-treated oxygen-delignified pine kraft pulp lignin. On the other hand, oxygen delignification had a smaller effect on carbohydrates, the main difference being the decrease in galactose content, which agrees with earlier results (*19*). The results may thus indicate that some of the linkages between lignin and carbohydrates were cleaved during laccase/NHA treatments, which may partly explain the improvement in the bleachability of laccase/mediator-treated pulps *(3,4,5)*.

Table II. Yields and Nitrogen, Protein and Total Carbohydrate Contents and Monosaccharides (After Acid Hydrolysis) of Residual Lignins of the Pulps Shown in Table I.

Res.lignins	*Kraft*	*LE*	*(L/NHA)E*	*OO*	*OOLE*	*OO (L/NHA)E*
Yield, %[a]	96	109	110	92	91	77
N, %	0.49	0.61	1.89	2.30	2.20	2.47
Protein, %	3.1	3.8	11.8	14.4	13.8	15.4
Carbohydr., mg/100mg[b]	7.51	8.22	6.98	7.46	7.33	6.28
Man, mg/100mg	2.31	2.52	1.78	2.13	2.25	1.60
Xyl, mg/100mg	1.68	2.09	1.91	1.97	1.91	1.79
Glc, mg/100mg	1.43	1.56	1.44	1.90	1.88	1.66
Gal, mg/100mg	1.72	1.61	1.47	0.97	0.83	0.80
Ara, mg/100mg	0.37	0.43	0.39	0.49	0.46	0.43

[a] Yield of isolated lignin as % of pulp lignin content; pulp lignin content calculated by multiplying lignin kappa number by 0.15.

[b] Corrected for carbohydrates originating from cellulases.

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Functional Groups of the Residual Lignins

The FTIR spectra of residual lignins obtained by subtracting the FTIR spectrum of the protein impurity from the spectra of the original residual lignins are shown in Figure 2. The most useful region in lignin analysis is the carbonyl region at 1750-1650 cm^{-1}, which gives information about changes in the oxidation degree in relation to the aromatic bands at 1500 cm^{-1}.

The FTIR spectra of the residual lignins show that laccase/NHA treatment increased the intensity ratio of the carbonyl band at 1720 cm^{-1} to that of the aromatic band at 1510 cm^{-1} indicating an increase of carboxylic acid or ester groups in relation to aromaticity. Laccase/NHA treatment of kraft pulp also increased the intensity of the band at 1660 cm^{-1} in relation to the aromatic band, indicating formation of conjugated carbonyl structures such as quinone structures. Similar increases in carboxylic and carbonyl groups have been seen to occur on laccase/HBT treatment (*20*). Unconjugated carbonyl groups in aldehydes and ketones would also stretch in this region.

The phenol content of the residual lignin of the pine kraft pulp and the oxygen-delignified pine kraft pulp decreased by about 30% during laccase/NHA treatment, indicating that free phenolics are reactive sites in delignification (Figure 3). The results thus agree with those obtained for other types of pulps (*18,21*) and for HBT-mediated delignification (*5,20*). Model compound studies, however, have shown that laccase in the presence of mediator also degrades non-phenolic structures (*22,23*). The increase in the proportion of conjugated phenols due to the formation of α-carbonyl groups was similar to that brought about by laccase in the presence of HBT. As with other laccases (*20*), ***Trametes versicolor*** left the fiber phenols intact, probably because of the fiber matrix.

Increasing the NHA charge from 48 to 74 mmol/kg pulp did not affect the concentration of free phenols in the residual lignins of oxygen-delignified pine kraft pulp. Comparing the effects of NHA and HBT as a mediator showed that the contents of phenolic hydroxyl groups in residual lignins were equal when equal amounts of mediator (on a molar basis) were used. These results agree well with the delignification efficiencies obtained (Figure 1). In another investigation it was found that laccase in the presence of NHA lowered the concentration of free phenols in the residual lignin of softwood kraft pulp slightly more than laccase in the presence of HBT (*18*).

The decrease in the methoxyl group content of pulp lignins with increasing delignification degree (Figure 4) was much smaller than the decrease in aromatic structures (Figure 5). The results thus show that cleavage of aromatic rings occurred without simultaneous demethylation.

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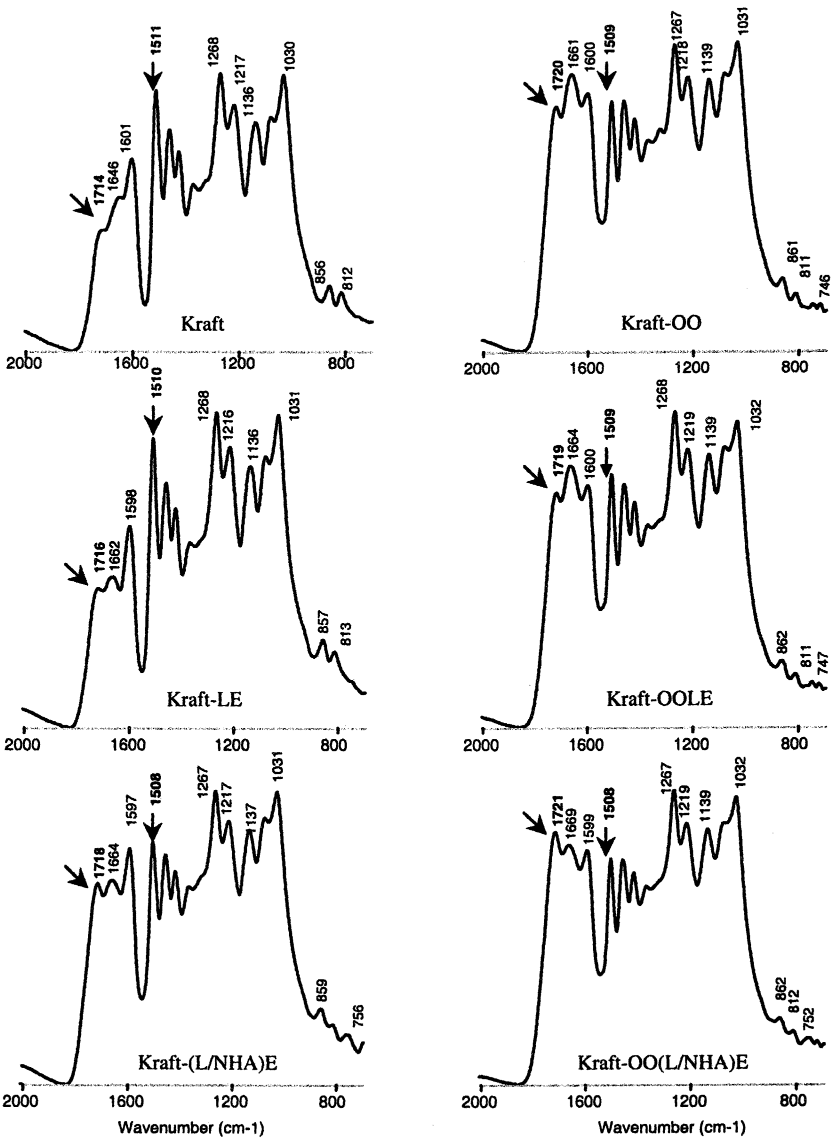

Figure 2. Difference FTIR spectra of residual lignins (protein impurities have been subtracted) from pine kraft pulp, laccase-treated and laccase/NHA-treated pine kraft pulps after alkaline extraction, and from oxygen-delignified pine kraft pulp before and after laccase or laccase/NHA treatment followed by alkaline extraction.

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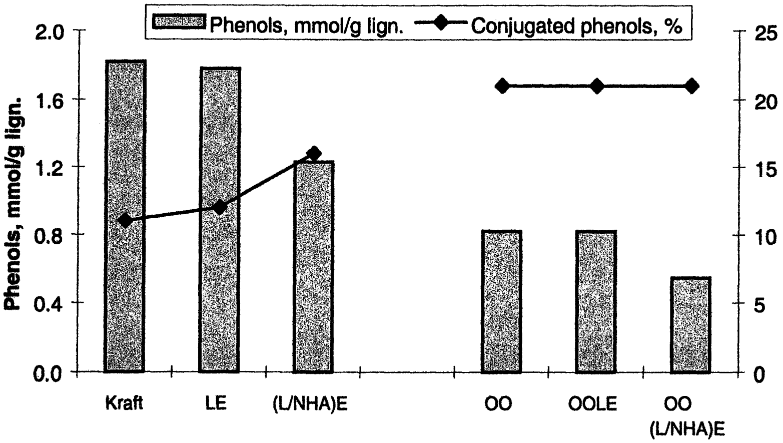

Figure 3. Total phenols and conjugated phenols (% of total) in residual lignins of pine kraft pulp, laccase-treated and laccase/NHA-treated pine kraft pulps after alkaline extraction, and from oxygen-delignified pine kraft pulp before and after laccase or laccase/NHA treatment followed by alkaline extraction. (Values corrected for protein impurities and carbohydrates).

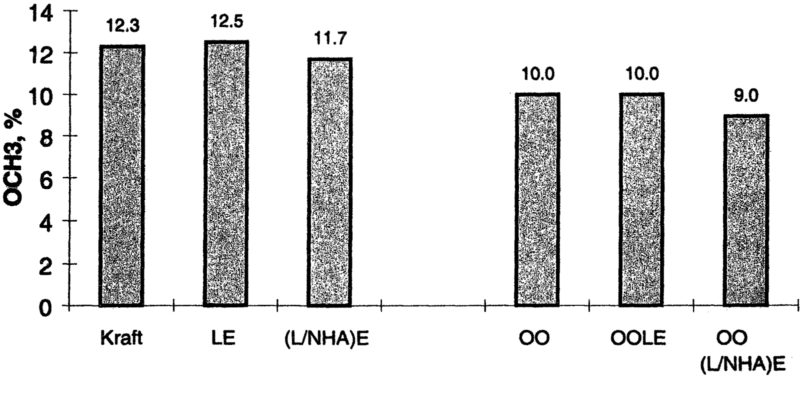

Figure 4. Methoxyl groups in residual lignins of pine kraft pulp, laccase-treated and laccase/NHA-treated pine kraft pulps after alkaline extraction, and from oxygen-delignified pine kraft pulp before and after laccase or laccase/NHA treatment followed by alkaline extraction. (Values corrected for protein impurities and carbohydrates).

Lignin Structure Analysed by Analytical Pyrolysis

The isolated residual lignins were weighed (80 μg) and subjected to pyrolysis. A total of 19 monomeric compounds representing guaiacyl (G) and *p*-hydroxyphenyl (H) lignin degradation products were separated and identified by GC/MS (*13*). The peak areas were quantified and normalized to the weight of sample. Repeatability was 10% (RSD) calculated from the peak areas of dublicate samples. A black liquor lignin sample was analysed similarly in the same series and used as an external standard representing totally aromatic lignin.

The peak areas for the residual lignin of pine kraft pulp and the external standard were equal, showing that these lignins have the same aromaticity. Laccase treatment did not change the content of aromatic residual lignin, as shown in Figure 5. However, the residual lignins of both oxygen-delignified pulp and laccase/NHA-treated pine kraft pulp contained significantly lower concentrations of aromatic subunits. After laccase/NHA treatment, the aromaticity of the residual lignin from oxygen-delignified pulp was further diminished. These results clearly show that laccase/NHA treatment oxidized aromatic residual lignin in the pulps to non-aromatic lignin structures such as muconic acids. The change caused by oxygen was very similar to that brought about by laccase/NHA. Only 60% of the residual lignin in the oxygen-delignified pulp and the laccase/NHA-treated kraft pulp retained its aromatic structure. Less than 50% of the residual lignin in the laccase/NHA-treated oxygen-delignified pulp was aromatic (Figure 5).

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The less aromatic nature of the lignins was not, however, seen as a decrease in UV absorptivity. On the contrary, the absorptivities of the laccase/NHA-treated samples were even higher than those of the untreated, as seen in Figure 5. The formation of highly UV-active structures like quinones could explain this phenomenon.

On the other hand, the composition of the aromatic pyrolysis products showed that there are only insignificant differences in the chemical structures of the aromatic parts of the different isolated residual lignins (Figure 6). Laccase and laccase/NHA treatment, however, caused a small increase in the content of α-carbonyl structures, which agrees with the results shown in Figures 2 and 3. The increase in α-oxidized structures was greater with oxygen treatment than with laccase/mediator treatment.

Pyrolysis gives information about residual lignin structures without the need to isolate the residual lignin from the pulp. Direct pyrolysis analysis of the pulps showed a significant enrichment of *p*-hydroxyphenyl structures caused by laccase/NHA treatment, indicating their stability against mediator-aided delignification (Figure 7). The enrichment of *p*-hydroxyphenyl structures seen in the isolated residual lignins was insignificant (Figure 6). The probable reason for the difference in the concentration of *p*-hydroxyphenyl units obtained by pyrolysis of pulp and isolated lignins is that *p*-hydroxyphenyl lignin was

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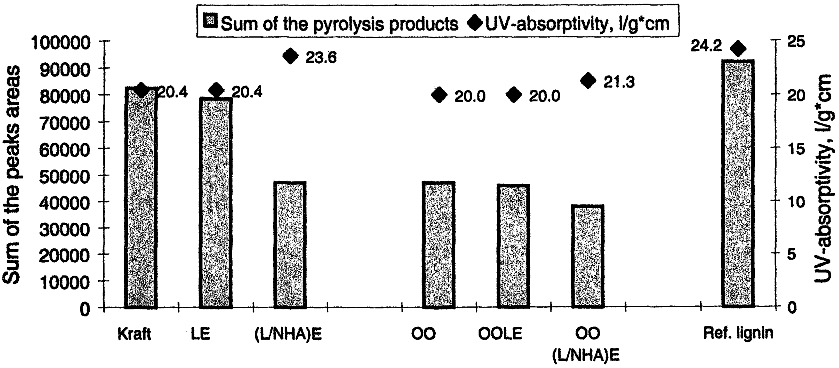

Figure 5. Sum of the pyrolysis products showing the content of aromatic lignin in residual lignins of pine kraft pulp, laccase-treated and laccase/NHA-treated pine kraft pulps after alkaline extraction, and from oxygen-delignified pine kraft pulp before and after laccase or laccase/NHA treatment followed by alkaline extraction. The absorptivities of the residual lignins are included in the Figure. A black liquor lignin sample (reference) was used as an external standard.

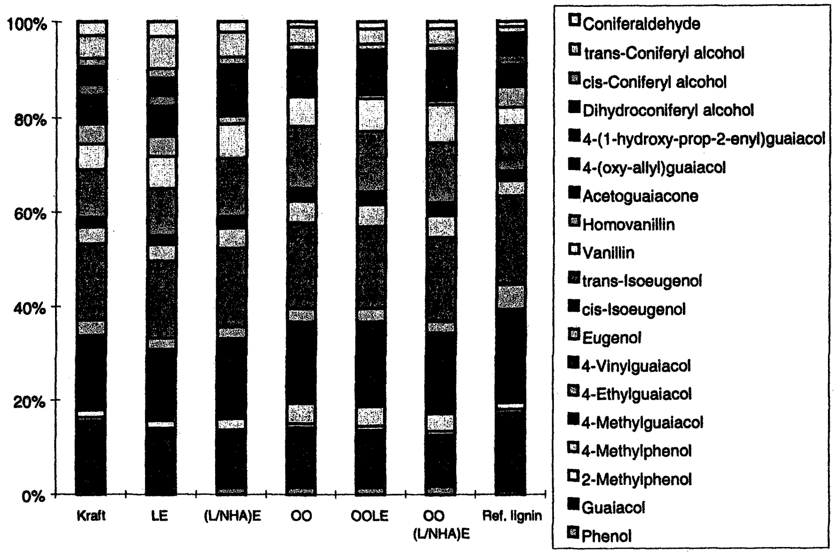

Figure 6. Composition of the pyrolysis products in the residual lignins of pine kraft pulp, laccase-treated and laccase/NHA-treated pine kraft pulps after alkaline extraction, and from oxygen-delignified pine kraft pulp before and after laccase or laccase/NHA treatment followed by alkaline extraction. A black liquor lignin sample (reference) was used as an external standard.

precipitated in the isolation procedure to a lower extent than guaiacyl lignin (*24*). The increase in *p*-hydroxyphenyl content agrees with that obtained for birch pulp, which showed that the syringyl structures were more reactive than guaiacyl structures and that *p*-hydroxyphenyl structures were stable against laccase/HBT treatment (*25*).

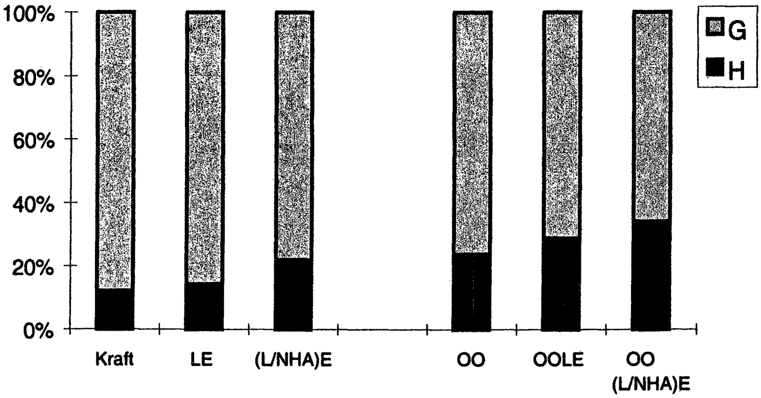

Figure 7. Proportion of guaiacyl (G) and p-hydroxyphenyl (H) structures in pine kraft pulp, laccase-treated and laccase/NHA-treated pine kraft pulps after alkaline extraction, and from oxygen-delignified pine kraft pulp before and after laccase or laccase/NHA treatment followed by alkaline extraction.

Fate of NHA during delignifcation

NHA-derived products were found to be linked to lignin, as two degradation products originating from NHA were identified in the pyrograms of the lignins from the laccase/NHA-treated pulps. The products were aniline and acetanilide, the reduced form of NHA. Aniline and benzotriazole, the reduced form of HBT, have previously been detected in corresponding laccase/HBT treated samples (unpublished results). Thus, these two seem to react similarly. The degradation products were clearly visible in the pyrograms. Still, their contents were probably very low, because the nitrogen contents of the samples corresponded, with reasonable accuracy, to their protein contents as determined with the pyrolysis method.

Conclusions

Delignification with the laccase/NHA system induced structural changes in pulp residual lignin that were very similar to those found previously for

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laccase/HBT treatment. The most reactive functional group seems to be the phenolic hydroxyl group, the number of which was reduced during the treatment. A simultaneous increase in the proportion of α-conjugated phenols was observed. The increase in carboxylic acid groups was seen in the FTIR spectra of the isolated residual lignins and also as an increase in the total acid content in the case of the lignin-rich unbleached kraft pulp.

Using analytical pyrolysis in a quantitative way, the content of aromatic lignin structural units was found to be much lower in the delignified samples, although their distribution was fairly similar in all cases. This can be explained by the conversion of aromatic units into aliphatic structures like muconic acids and/or by the formation of structures such as quinones. The FTIR spectra supported the presence of quinones, which could also explain the high UV absorptivities of these samples.

Carbohydrates, including hexenuronic acid, were intact. However, the content of lignin-bound carbohydrates in the laccase/NHA-treated samples was slightly lowered, indicating a reduction in the number of lignin-carbohydrate linkages.

Residues of NHA degradation products were detected in the isolated lignins by pyrolysis. These were identified as aniline and acetanilide.

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Chapter 23

On the Role of 1-Hydroxybenzotriazole as Mediator in Laccase Oxidation of Residual Kraft Lignin

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In order to elucidate the role of the mediator 1-hydroxybenzotriazole (HBT) in laccase oxidation processes, vanillyl alcohol was oxidized with laccase in the presence and in the absence of this mediator. The pattern of metabolites produced showed that the presence of HBT allowed the formation of oxygen addition products such as *o*- and *p*-quinones and aromatic ring cleavage products, while in its absence only oxidative coupling products were observed. Experiments carried out on residual kraft lignin showed the occurrence of coupling reactions during laccase oxidation. In the presence of the laccase-mediator (LM) system the reaction pathway was driven toward side-chain oxidation and oxygen addition products, while the formation of condensed structures was depressed. Furthermore, The role of phenolic, aliphatic OH, and COOH moieties in lignin degradation was elucidated by selectively blocking them and then submitting the samples to laccase and laccase-HBT treatments. It was found that the activity of the LM system depends on the presence of phenolic lignin subunits. In actual fact, the reactions carried out on lignins lacking free phenolic groups did not cause any appreciable degradation to the polymer.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Introduction

In Nature only few microorganisms are able to perform wood rotting. Among them the white-rot basidiomycetes produce mixtures of exocellular enzymes (oxidases and peroxidases) which are able to degrade lignin in wood. Lignin peroxidase, manganese peroxidase and laccase have all been studied in order to clarify the mechanism of natural wood degradation (1). Possible industrial applications in processes of low environmental impact such as pulping and bleaching for paper making have prompted further interest in their use (1). Laccase, benzenediol:oxygen oxidoreductase 1.10.3.2 is a multicopper oxidase which performs a one-electron oxidation on a variety of aromatic substrates (2). This enzyme displays a high thermal resistance (stable at 60 °C) (3) and low substrate specificity being able to oxidize a number of different aromatic substrates. Its lack of substrate inhibition coupled with its high oxidation rates (10-100 fold higher than those of Lignin peroxidase or Manganese peroxidase) make laccase an ideal candidate for the development of enzymatic pulping processes (3-5). There are many studies dealing with the laccase oxidation mechanism (5-10). It is generally assumed that its catalytic activity occurs through the generation of a phenoxy radical on the phenolic substrate by a one-electron oxidation and hydrogen (H+) abstraction process (Scheme 1). The oxidation of non-phenolic substrates is prevented by their high redox potential.

However, laccase itself has a poor effect on pulps, while when used in the presence of radical mediators such as 1-hydroxybenzotriazole (HBT) it has been shown to effectively demethylate kraft pulps (11). Consequently, extensive efforts have been made to understand the mechanism of the laccase-mediator (LM) system. Several reports have appeared in the literature attempting to deal with the LM oxidation of non-phenolic compounds; the increased delignification of the LM systems being ultimately ascribed to their ability to effect the oxidation of non-phenolic lignin subunits (11-19). More specifically in the presence of the LM system it has been shown that low amounts of oxidation of non-phenolic β-O-4 aryl ether model compounds can occur (12).

Despite the large body of information that exists on the reaction of laccase with several substrates and lignins, to date a comprehensive interpretation of the reaction mechanism of the LM system is still lacking. In fact, since the early finding that laccases in the presence of radical mediators are able to bring about the oxidation of some non-phenolic model compounds, little or no attention has been devoted to their behavior toward phenolic lignin subunits (20).

In an effort to further comprehend the mechanism of activity of the radical mediators ABTS and HBT on laccase oxidations, in a previous communication (21) we selected an array of model compounds resembling the fundamental bonding patterns of residual kraft lignin. The experiments were performed on 5-5', diphenylmethane, 4-O-5' and stilbene phenolic model compounds, structures that constitute the bulk of residual kraft lignin (22-25). Reactions with laccase and laccase + HBT or laccase +ABTS (2,2'-azinobis-(3-ethyl-benzthiazoline-6-sulfonate)) showed that in the presence of HBT or ABTS only side-chain

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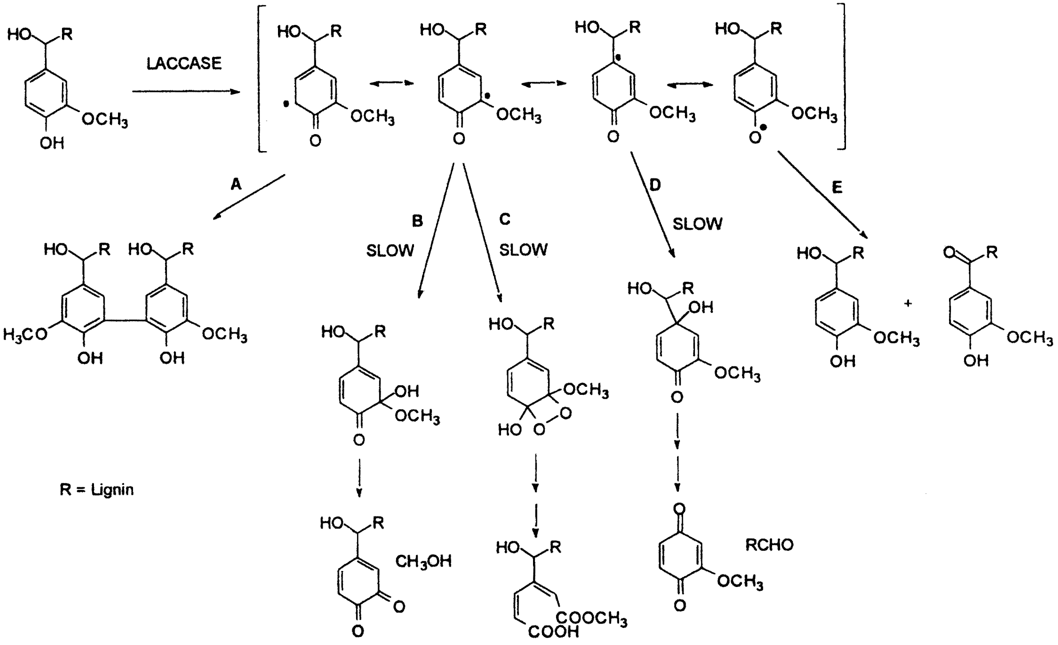
LACCASE
A
B
SLOW
C
SLOW
D
SLOW
E
R = Lignin
CH₃OH
RCHO
HO
R
OH
OCH₃
H₃CO
O
COOCH₃
COOH

Scheme 1. Generally proposed reaction pathway for the laccase oxidation of lignin

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oxidation reactions and demethoxylations occurred (21). It is significant to note that under the reaction conditions chosen it was not possible to identify polymerization products. Alternatively the oxidation of the corresponding non-phenolic compounds did not occur. On the basis of these data we suggested that the role of radical mediators is to act as diffusible lignin oxidizing agents of phenolic systems, since such compounds can access the inner lignin structure with greater facility than the enzyme itself (21).

In an effort to further elucidate the role of phenolic or non-phenolic lignin subunits in the LM system we designed a set of experiments aimed at clarifying this issue.

More specifically vanillyl alcohol was used as monomeric model compound. It was oxidized with laccase in the presence and in the absence of HBT. The role of phenolic, aliphatic OH, and COOH moieties in lignin degradation was elucidated by selectively blocking each of them by diazomethane methylation, acetylation, and acetylation followed by alkaline hydrolysis and the modified samples were then submitted to laccase and laccase-HBT treatment.

The modifications induced on the lignin polymer were determined by means of quantitative ^{31}P-NMR of lignin samples suitably phosphytilated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxphospholane (26-31). This methodology provided a clear insight on the residual kraft lignin structural modifications and permitted the clarification of the role of HBT during the oxidation.

As such, it became possible to determine that the LM system is able to modify the content and distribution of phenolic OH, aliphatic OH and COOH groups only when the lignin sample possesses free phenolic groups. On the basis of our data it was possible to establish the role of HBT in laccase oxidations in driving the reaction pathway toward HBT radical mediated oxidation to side-chain oxidation and oxygen addition products rather than to oxidative coupling reactions.

Results and Discussion

Oxidation of vanillyl alcohol

Vanillyl alcohol **1**, a phenolic substrate, was submitted to laccase oxidation in the presence and in the absence of HBT. After ten minutes, the reaction mixtures were acidified, extracted with ethyl acetate and the residues were analyzed by quantitative GC-MS analyses in the presence of a suitable amount of 2,4-dimethoxy toluene as an internal standard.

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The % conversions of vanillyl alcohol under the different reaction conditions are shown in Table I and scheme 2. It is evident that the presence of HBT did not significantly affect the conversion amount. However, the distribution of the metabolites produced under the two conditions is fundamentally important for the interpretation of the operating mechanisms. Unfortunately, under our experimental conditions the bulk of reaction products were not identified due to their high molecular weight (> 550). In fact, in a control experiment all the reaction mixtures were freeze dried and the residue was submitted to silylation and GC-MS analyses.

This experimental protocol showed that no further low molecular weight reaction products were lost during the reaction workup, with the exception of volatile one carbon fragments such as methanol.

In the presence of laccase alone, only traces of vanillin **2**, a product of side chain oxidation, and a low amount of **3**, a product rising probably from radical coupling, were detected. In contrast when the LM system was used in the oxidation of **1**, the reaction pathway is shown to be more complex. The presence of HBT in the reaction mixture induced a major change in the mechanism, yielding *o*-quinone **4**, *p*-quinone **5**, muconic acid **6** and phenol **7** (Scheme 2).

It is assumed that laccase catalyzes the reduction of oxygen to water with the generation of a phenoxy radical from a phenolic substrate by a one electron oxidation process and a proton loss. The phenoxy radicals generated by the laccase mediated oxidation of phenolic lignin subunits, in its early steps, can in principle undergo several different reactions. More specifically, radical coupling reactions yield condensed products (Scheme 1A). Further oxidation / oxygen addition in the C-3 position yields *o*-quinones and methanol (Scheme 1B); with the eventual aromatic ring cleavage via dioxetane intermediates (Scheme 1C) (5). Oxidation in C-1 yields alkyl-aryl cleavage with *p*-quinone formation (Scheme 1D) (5). However since phenoxyl radicals do not react with molecular oxygen at any noticeable extent (32,33) ($k< 10^{-2}$ $M^{-1}sec^{-1}$) the observed formation of quinones and aromatic ring cleavage products should be brought about by superoxide anion radicals reacting with phenoxy radicals (34). It is likely that the phenoxy radical intermediates may also undergo disproportionation reactions yielding side-chain oxidation products (Scheme 1E). In the presence of laccase and HBT it is assumed that the HBT radical is generated (35). This can react with lignin phenolic subunits *via* hydrogen abstraction regenerating HBT (Scheme 3).

In principle, hydrogen abstraction can occur either at the phenolic or at the benzylic position (Scheme 3). In the former case a phenoxy radical would be formed. As such HBT would act as a diffusible radical mediator. If hydrogen abstraction occurs at the benzylic position a benzylic radical would be formed.

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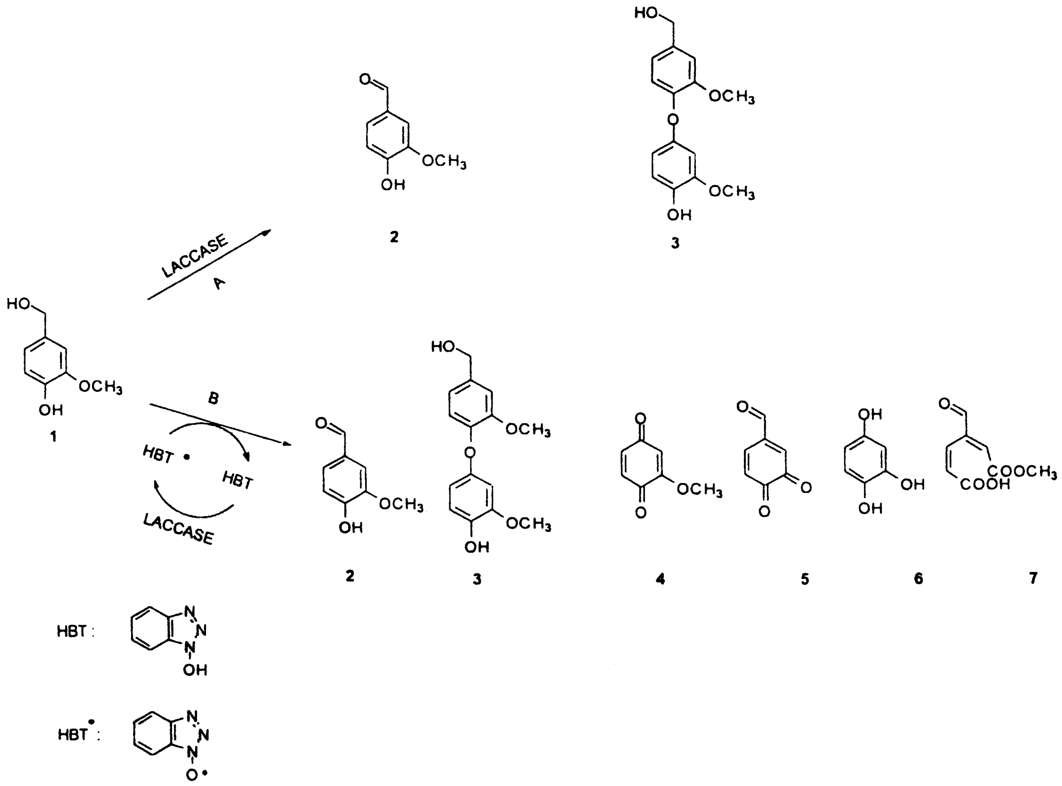
LACCASE
A
B
HBT •
HBT
LACCASE
HBT :
HBT• :
1
2
3
2
3
4
5
6
7

Scheme 2. Products detected after laccase (A) and laccase + HBT (B) treatment

Table I. Product identity and yields detected after laccase and laccase + HBT treatments of vanillyl alcohol

Product	LaccaseTreatment (% of the starting product)	Laccase + HBT Treatment
1	14.8	12.4
2	traces	traces
3	0.8	1.3
4	-	traces
5	-	4.0
6	-	4.0
7	-	6.9

The products missing from the mass balance have been tentatively assigned as radical oxidative-coupling polymeric products and/or volatile one-carbon fragments.

This intermediate is likely to undergo rapid oxygen addition and after loss of superoxide anion radical, to yield the side-chain oxidation products shown in Scheme 3. It has been shown that benzylic radicals are reactive toward oxygen addition at rates of 10^{10} $M^{-1}sec^{-1}$. The superoxide radicals formed during this step would in turn readily react with phenoxy radicals thus allowing the formation of further oxidation products.

Attack at the 3 position would cause the formation of *o*-quinones and methanol, while addition in the 4 position ultimately yields aromatic ring cleavage products (muconic acids); finally addition in position 1 followed by aryl-alkyl cleavage results in the formation of *p*-quinones (Scheme 3). This reaction mechanism could explain the formation of products **3-6** from the LM system on vanillyl alcohol.

The preceeding is in accord with the previously reported laccase mediated oxidations of lignins in which the formation of condensed units, quinones, methanol, aryl-alkyl cleavage, side-chain oxidation and aromatic ring oxidation products has been reported (5, 11, 14, 36, 37).

Oxidation of residual kraft lignin

Residual kraft lignin (RKL) was oxidized in the presence of either laccase or laccase+HBT. The lignin samples isolated after the treatments were phosphytilated and then submitted to ^{31}P-NMR analyses in the presence of a suitable amount of cyclohexanol as an internal standard. The assignment of the different signals was carried out on the basis of earlier work (29, 30, 38).

All experiments were carried out in triplicate and the quantitative data collected is reported in Table II. The analysis of the amount and distribution of aliphatic OH, phenolic OH and COOH groups showed a different oxidation pathway in the presence and in the absence of HBT. Irrespective of the presence or absence of HBT the aliphatic OH and the guaiacyl phenolic OH groups were

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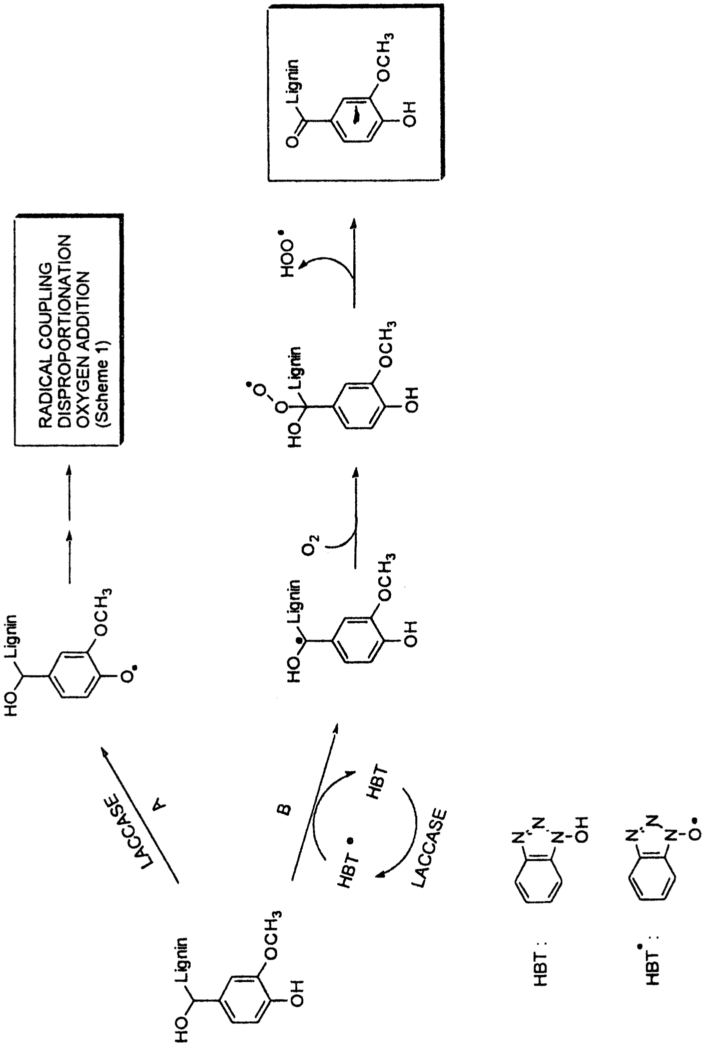
RADICAL COUPLING
DISPROPORTIONATION
OXYGEN ADDITION
(Scheme 1)
LACCASE
A
B
HBT
HBT •
LACCASE
O_2
HOO•
Lignin
OCH_3
HO
OH
HBT :
HBT• :

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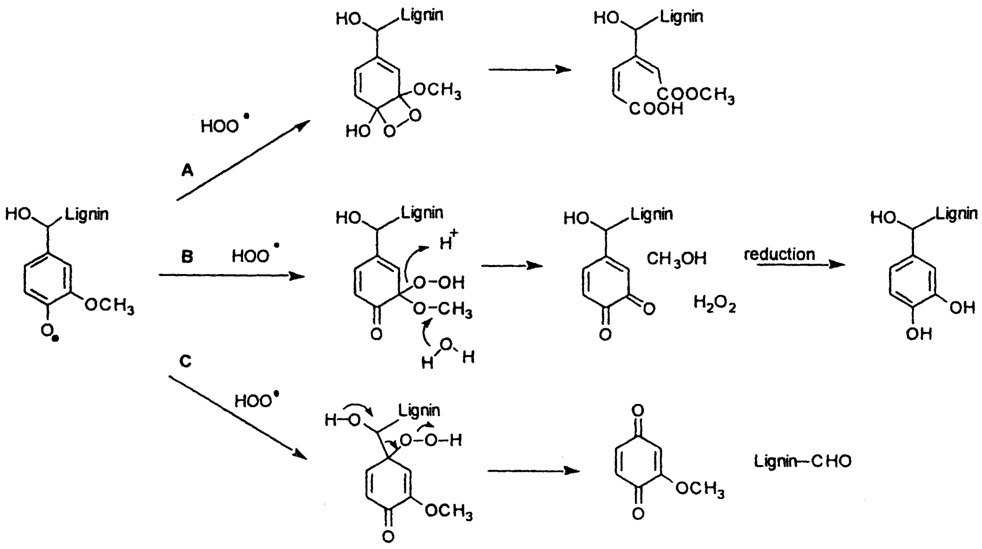
A
HOO•
B
HOO•
C
HOO•
HO
Lignin
OCH₃
O•
HO
Lignin
OCH₃
HO
O
O
COOCH₃
COOH
H⁺
O-OH
O-CH₃
O
H
H
CH₃OH
H₂O₂
reduction
OH
OH
H-O
O-O-H
Lignin-CHO

Scheme 3. Proposed reaction pathway for the oxidation of lignin by the laccase-HBT system found to be in lower amounts than in the original RKL, despite the fact that the decrease was more remarkable in the presence of HBT. This is in agreement with the previously hypothesized role of HBT in lignin degradation. The COOH content was found to increase by 20%, (from 0.34 to 0.41 mmole/g), upon laccase treatment. When HBT was present in the reaction mixture no variation in COOH content was evident. The corresponding decrease of guaiacyl OH, condensed phenolic OH and aliphatic OH groups could be due to the loss of small oxidized fragments.

The distribution of the condensed phenolic units present in residual kraft lignin in different reaction conditions was significantly modified. The diphenylmethane substructures (^{31}P-NMR range 144.3-142.8 ppm) (39) were found to increase upon laccase treatment. This could be due to either reaction with phenolate units or formation of one-carbon fragments. In contrast, when HBT was present in the reaction medium the amount of diphenylmethane did not significantly change. This indicates that the oxidative reactions occur via a different pathway. In the same fashion the abundance of 4-O-5' subunits (^{31}P-NMR range 142.75-141.75 ppm), which are formed by radical coupling reactions, were found to increase by 75%, (from 0.36 to 0.63 mmole/g), after the laccase treatment. In the presence of HBT, however, their abundance did not change. Condensed 5-5', units (^{31}P-NMR range 141.75-137.4 ppm) i.e. radical coupling products, were also found to be somewhat increased upon laccase treatment, and significantly decreased in the presence of HBT (Table II).

From these data a clear trend becomes apparent: the laccase and the laccase-mediator oxidation of residual kraft lignin (RKL) proceed through different reaction pathways. In the presence of HBT side-chain oxidation reactions are favored as opposed to radical coupling and condensation reactions.

When the treatment was carried out without HBT, the formation of condensed units occurred as the main reaction.

It is likely that HBT could act as a radical mediator by readily diffusing into the lignin's inner structure. Consequently HBT radicals generated by laccase oxidation could drive the overall reaction toward the Cα oxidation pathway via hydrogen atom abstraction reactions. The superoxy radicals formed in this step (Scheme 3) would be responsible for aromatic ring cleavage and quinone formation reactions.

Oxidation of lignin with selectively protected moieties

In an effort to establish the role of non-phenolic units during the laccase-HBT catalyzed oxidation of RKL a selective protection scheme for the various functional groups containing labile protons was devised (Scheme 4). More specifically RKL was submitted to exhaustive diazomethane methylation which allowed the selective methylation of the phenolic OH and COOH groups while leaving free the aliphatic OH groups. The quantitative ^{31}P-NMR spectrum of the

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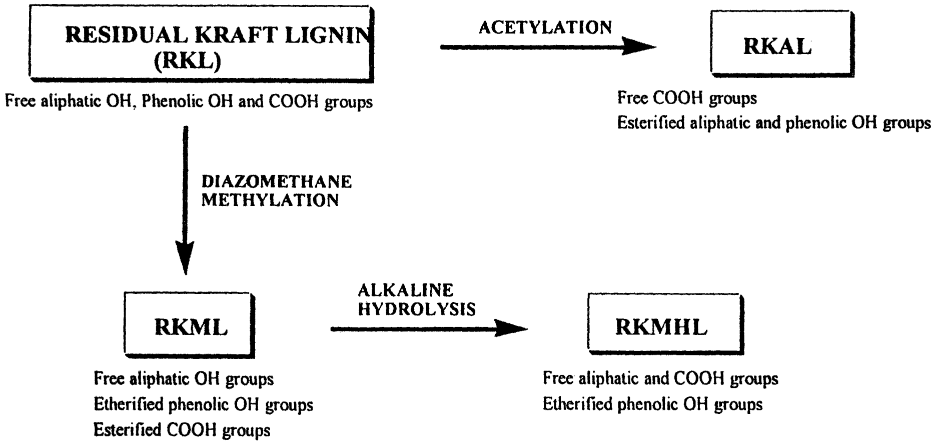
Table II. Phenolic, aliphatic and carboxylic OH groups (mmoles/g) present on residual kraft lignin before and after treatment with laccase and laccase + HBT determined by quantitative ^{31}P-NMR analysis

Functional group	*RKL[1] (mmole/g)*	*RKL + Laccase (mmole/g)*	*RKL + Laccase + HBT (mmole/g)*
Aliphatc OH	2.50	2.36	2.10
Diphenylmethane OH	0.23	0.31	0.22
4-O-5' condensed OH	0.36	0.63	0.35
5-5' Condensed OH	0.66	0.68	0.59
Guaiacyl OH	1.25	1.00	0.85
COOH	0.34	0.41	0.34

[1] Residual kraft lignin.

diazomethylated residual kraft lignin (RKML) after phosphytilation showed the effective presence of aliphatic OH groups only.

Diazomethylated residual kraft lignin was than treated with laccase and laccase + HBT respectively. The ^{31}P-NMR spectrum of the recovered samples after phosphytilation was found to be unaltered. In particular, neither condensed phenolic or guaiacyl OH groups were formed. In the presence of laccase alone, an increase in COOH units was apparent, most probably arising from residual acetate buffer. Neither laccase nor laccase+HBT were able to modify the distribution or the amount of aliphatic OH, phenolic OH and COOH groups in RKML.


RESIDUAL KRAFT LIGNIN (RKL)
Free aliphatic OH, Phenolic OH and COOH groups
ACETYLATION
RKAL
Free COOH groups
Esterified aliphatic and phenolic OH groups
DIAZOMETHANE METHYLATION
RKML
Free aliphatic OH groups
Etherified phenolic OH groups
Esterified COOH groups
ALKALINE HYDROLYSIS
RKMHL
Free aliphatic and COOH groups
Etherified phenolic OH groups

Scheme 4. Selective protection of phenolic, aliphatic and carboxylic OH groups.

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The role of COOH groups was studied by exposing diazomethylated RKL to alkaline hydrolysis (RKMHL)(Scheme 4). This scheme liberated the COOH and aliphatic OH groups while the phenolic OH groups were still protected. The ^{31}P-NMR spectrum obtained confirmed this functional group path. Accordingly, RKMHL was also submitted to treatment with laccase and laccase + HBT and it was then examined by quantitative ^{31}P-NMR spectroscopy. Once again, no degradation of the lignin polymer with respect to the control experiment was operable. Analogous behavior was found when RKL was submitted to extensive acetylation (RKAL). Under these conditions only COOH groups were free, as revealed by the ^{31}P-NMR spectrum. Neither laccase, nor laccase +HBT were able to modify the amount and/or distribution of the OH groups.

These data when coupled with our earlier studies on RKL model compounds, imply that the activity of the laccase-mediator system does not depend upon the nature of the non-phenolic lignin subunits. In actual fact, LM system reactions with lignin containing protected or unprotected phenolic, aliphatic and carboxylic acid groups, did not result in any appreciable lignin degradation. On the contrary, when the LM system was employed on RKL, HBT showed the ability to drive the degradation pathway toward side-chain oxidation products, and depressed the formation of condensed structures.

Our data indicates that in the presence of HBT the side-chain oxidation pathway is strongly favored over oxidative coupling reactions (40). Previous reports describe that the depolymerization of lignin occurs only when treated with laccase +HBT. Earlier accounts describe both an increase in carbonyl and quinone content, and a decrease in the aromatic unit content after lignin treatment with the LM system while in the absence of HBT no depolymerization were found (35, 36, 41). This is in agreement with the behavior of the laccase and LM system reported here. Analogously, the LM system provides pulps which are darker and can be more easily bleached with hydrogen peroxide than those obtained from treatments in the absence of a mediator. The darkness and higher bleachability may be due to an increased quinone content.

Conclusions

Experiments with vanillyl alcohol showed that in the presence of laccase only oxidative coupling occurs. However, in the presence of the LM system the formation of *o*- and *p*-quinones, demethylation, aromatic ring cleavage and oxidative coupling reactions was apparent.

The LM system is not operating when the phenolic OH groups in residual kraft lignin are protected.

Experiments carried out on residual kraft lignin showed the occurrence of coupling reactions during laccase oxidation. In the presence of the LM system the reaction pathway was driven toward side-chain oxidation and oxygen addition products, while the formation of condensed structures was depressed.

One may argue that HBT does not act as a mediator in the oxidation of non-phenolic lignin subunits. The principal sites of oxidative attack in the laccase/HBT system are the free phenolic groups in lignin (36). In our view HBT' is able to promote the oxidative reactivity of laccase toward side-chain oxidation processes. A possible mechanism is the benzylic hydrogen abstraction reaction. The benzylic radical is a species with a high degree of reactivity toward oxygen. Such an intermediate reacts by oxygen addition yielding a side-chain oxidation product with the simultaneous generation of superoxide anion radical (Scheme 3). In turn the superoxide anion radical reacts with the phenoxy radical by addition in the position 1, 3 or 4 of the aromatic ring. Thus the formation of side-chain oxidation, oxygen addition and aromatic ring cleavage products or substructures is favored and consequent 5-5' and/or 4-O-5' couplings are depressed.

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Experimental

Quantitative ^{31}P-NMR spectra were obtained on a Varian XL-300 spectrometer by using methods identical to those described by Argyropoulos *et al*. (26-31). The chemical shifts were referenced to phosphoric acid. The ^{31}P-NMR data reported in this effort are averages of three phosphitylation experiments followed by quantitative ^{31}P-NMR acquisitions. The maximum standard deviation of the reported data was 2.10^{-2} mmol/g, while the maximum standard error was 1.10^{-2} mmole/g.

Derivatization of the lignin samples with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (II) (27, 29, 30, 39) were performed as previously described. Samples of lignin, (30 mg) accurately weighed, were dissolved in a solvent mixture composed of pyridine and deuterated chloroform, 1.6:1 v/v ratio (0.5 mL). The phospholane (100 µL) was then added, followed by the internal standard and the relaxation reagent solution (100 µL each).

Isolation of residual kraft lignin

Residual kraft lignin was isolated from kraft pulp (*picea mariana*, kappa no. 31.5) using a slightly modified acidolysis procedure (42, 43). The yield was 38%, and the purity was confirmed by UV and klason lignin content measurements.

Lignin methylation

Lignin (200 mg) was suspended in 5 mL of diethyl ether and then treated with an excess of diazomethane for 24 h in the dark at room temperature. The treatment was repeated three times. The mixture was centrifuged, washed with ethyl ether, and centrifuged again. The residue was dried under reduced pressure (27).

Lignin hydrolysis

Lignin (100 mg) was dissolved in 10 mL of 2M NaOH and stirred under a nitrogen atmosphere at 25°C for 48 h. 4 M HCl was then added to obtain pH 3 and the mixture centrifuged, washed with water, centrifuged again and freeze-dried (27).

Lignin acetylation

Acetylation was carried out with pyridine/acetic anhydride (1:1) at 25°C for 48 h. 4 M HCl was then added to obtain pH 3 and the mixture was stirred 12 h. The residue was centrifuged, washed with water, centrifuged again and freeze-dried (27).

Enzyme assays

Laccase activity was determined by oxidation of ABTS. The assay mixture contained 0.5 mM ABTS, 0.1 M sodium acetate, pH 5.0, and a suitable amount of enzyme. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε_{420} = 3,6 x 104 $M^{-1}cm^{-1}$). Enzyme activity was expressed in units (U = mmol of ABTS oxidized per minute) (44).

Characterization of metabolites

Gas chromatography and gas chromatography-mass spectrometry of the reaction products were performed using a DB1 column (30 m X 0.25 mm and

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0.25 mm film thickness), and an isothermal temperature profile of 80°C for the first two min, followed by a 5°C/min temperature gradient to 200°C and a 15 °C/min gradient to 280°C and finally an isothermal period at 280°C for 10 min. The injector temperature was 250°C. Chromatography grade helium was used as the carrier gas. The fragmentation patterns were compared to those of authentic samples. The GC-MS fragmentation patterns are reported in Table III.

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Table III. Mass spectrometric data

Product	*Derivative*[a]	*MS (m/z) data (%)*
2	-	152 (M, 100), 151 (95), 109 (65), 81 (97).
3	$-Si(CH_3)_3$	418 (M, 4), 403 (2), 387 (3), 343 (5), 281 (5), 207 (23), 147 (6), 96 (11), 75 (26), 73 (100).
4	-	138 (M, 70), 123 (11), 110 (68), 108 (100), 95 (75), 82 (63), 69 (98), 55 (46), 54 (69).
5	-	136 M, 45), 121 (34), 104 (41), 79 (30), 75 (100).
6	$-Si(CH_3)_3$	315 (M+1, 6), 314 (M, 20), 207 (17), 131 (24), 129 (16), 117 (80), 111 (18), 106 (11), 97 (13), 85 (14), 82 (20), 75 (93), 73 (100).
7	$-Si(CH_3)_3$	342 (M, 5), 145 (13), 132 (30), 129 (19), 117 (68), 83 (11), 75 (67), 73 (100).

a -: underivatized; $-Si(CH_3)_3$: trimethylsilylated with N,O-bis(trimethylsilyl)-acetamide.

Laccase treatments of vanillyl alcohol 1

The oxidation was performed in a 1:3 (v/v) dioxane:water solvent mixture. The substrate (2 mM) was dissolved in dioxane: acetate buffer pH 5 0.05M (4 mL, 1:3 v/v), in the presence or absence of HBT (1mM), and treated with purified laccase from *Trametes versicolor* 0.5 U/mL at 40°C. The reaction mixtures were kept in open vials under vigorous stirring in order to ensure constant oxygen saturation of the solutions throughout the experiment[5]. The reactions were conducted in the dark in order to avoid possible photochemical oxidation. After 10 min the reactions were stopped by acidification with hydrochloric acid; a suitable amount of 2,4-dimethoxy toluene was then added to act as an internal standard (2mM), dissolved in dioxane. The reaction mixtures were extracted with ethyl acetate. The solutions were dryed over anhydrous $MgSO_4$ and filtered. The organic solvent was evaporated under reduced pressure. In order to analyze the reaction products the residues were dissolved in 1 mL of pyridine and silylated with N,O-bis(trimethylsilyl)-acetamide. After 30 min the mixtures were subjected to gas chromatography (GC) and gas chromatography-mass spectrometric (GC-MS) analyses.

Laccase treatments of residual kraft lignin

Lignin (100 mg) was suspended in acetate buffer pH = 5 0.05 M (10 mL) in the presence or absence of HBT (1 mM) and treated with with 10 U/mL at 40°C of purified laccase from Trametes versicolor supplied by Dr.Liisa Viikari of the VTT, Biotechnology and Food Research Laboratory, Finland. The reaction vessel was purged with oxygen. Oxygen was then applied until a positive pressure was obtained. After 24 h the mixture was cooled, acidified at pH 3 with acetic acid and centrifuged. The residue was washed with water three times, and then freeze-dried.

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Chapter 24

Development of Stable Redox Complexes to Mediate Delignification of Kraft Pulp by Laccase

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The use of laccase and mediators to delignify and bleach kraft pulp is now well documented. Several nitrogen-containing aromatic compounds have been shown to mediate the oxidation of lignin by laccase. In order to apply this technology in the kraft pulp industry, development of cost-effective mediators and laccases are of prime importance. Our electrochemical studies on the interaction between laccase, mediator and lignin indicate that effective mediators must behave like true catalysts, i.e. they must be reversibly reduced by lignin so that the mediator will be continuously regenerated and made available for further cycles of oxidation by laccase. We report on the use of transition metal complexes in combination with laccase to mediate catalytic delignification of kraft pulps. Due to their redox properties and stability, these metal complexes can be recycled many times for pulp delignification.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Introduction

Over the last ten years there has been a considerable research effort aimed at application of laccase and mediators in kraft pulp delignification and bleaching. This activity was initiated by the discovery (1,2) that several laccase artificial substrates, such as 2,2' azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) and 1-hydroxybenzotriazole (HBT), could allow the enzyme to catalyze delignification of kraft pulp. We called these substrates mediators, since they appear to act as electron carriers from lignin in the kraft fibre secondary wall to the enzyme which, due to its size, must reside outside the fibre (Figure 1). The laccase mediator system is capable of oxidizing both phenolic and non-phenolic subunits of lignin (3-9), but the rate and extent of delignification depend upon the redox potential of the laccase and mediator being used, and the lignin content of the pulp (10-14).

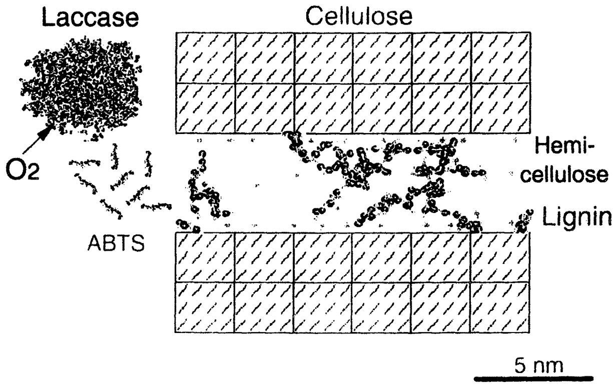

Figure 1. Oxidation of residual lignin in kraft pulp fiber with laccase and mediator

Properties of laccase mediators

The cost, dosage, and environmental impact of the mediator (which cannot practically be recovered) are crucial factors in successful application of laccase/mediator pulp bleaching. As a result of extensive screening, a growing number of nitrogen containing compounds have been found to mediate pulp delignification with laccase. Apart from ABTS and HBT, they include 1-nitroso-2-naphthol-3,6-disulfonic acid (NNDS) and 2-nitroso-1-naphthol-4-sulfonic acid (HNNS) (10,11,15), phenothiazines (16), violuric acid (11,17) and

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N-hydroxyacetanilide (NHA) (11,18). Recently we proposed to use transition metal complexes in combination with laccase to mediate the catalytic delignification and bleaching of kraft pulps (19,20).

The best known mediators are listed in Table I, together with their redox potentials, oxygen uptake rates with *Trametes versicolor* laccase, and extent of pulp delignification. The degree of delignification depends to some extent on the redox potential of the mediator as determined by cyclic voltammetry (21). It is not dependent on the rate of oxidation of mediator by laccase (relative oxygen uptake rate). Mediator stability, diffusion rate and kinetics of electron transfer with lignin are other factors which will influence the efficiency of delignification.

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Table I. Properties of laccase mediators

Mediator	Redox potential Vs NHE[1] V	Relative O_2 uptake rate[2]	Pulp[3] Delignification %
ABTS	0.67 ; 1.09	100	37
HBT	1.04*	1	45
Promazine	0.77 ; 1.06*	59	18
HNNS	0.91* ; 1.10*	46	34
NNDS	0.82	46	29
Violuric acid	0.91	14	42
NHA	0.83* ; 1.01*	42	42
$K_4Mo(CN)_8$	0.75	37	43

[1] NHE: Standard Hydrogen Electrode. Where two values are shown, this indicates that two electron transfers were observed within the measured range.
[2] In the absence of pulp.
[3] Softwood oxygen delignified kraft pulp, kappa no = 15. Mediator applied at 1% on pulp followed by a peroxide reinforced alkaline extraction (Ep)
*Electrochemically irreversible, redox potentials are lower than the values shown.

Transition metal complexes as laccase mediators

Our electrochemical studies on the interaction between laccase, mediator and lignin (21), has indicated that effective mediators must behave like true

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catalysts, i.e. they must be reversibly reduced by lignin so that the mediator will be regenerated and made available for further cycles of oxidation by laccase. This implies that in addition to being a laccase substrate, the mediator should have the following electrochemical properties: a highly reversible redox cycle, and a redox potential in the range of lignin (0.5 to 1.2 volts vs NHE). Several transition metal complexes have such properties and some of them have been traditionally used to measure redox potentials of laccases (22,23). Figure 2 shows cyclic voltammograms of four transition metal complexes namely: potassium octacyanomolybdate ($K_4Mo(CN)_8$), potassium octacyanotungstate ($K_4W(CN)_8$), iron tris-4,4'-dimethylbipyridine (FeII(4,4'-dmbpyr)$_3$) and iron tris-o-phenanthroline (FeII(o-phen)$_3$). The typical reversible voltammetric curves for these metal complexes indicate that they form stable redox couples. Thus, both their reduced and oxidized forms (i.e. Mo^{4+} / Mo^{5+} ; W^{4+} / W^{5+} and Fe^{2+} / Fe^{3+}) are stable enough to exist in aqueous solution so that they can be available to undergo reversible redox reactions with laccase and lignin. The redox potentials (E^0) of the complexes are measured at the mid-point between their oxidation and reduction peaks: $K_4W(CN)_8$: 0.49V; $K_4Mo(CN)_8$; 0.75V; FeII(4,4'-dmbpyr)$_3$: 0.89V; FeII(o-phen)$_3$: 1.10V.

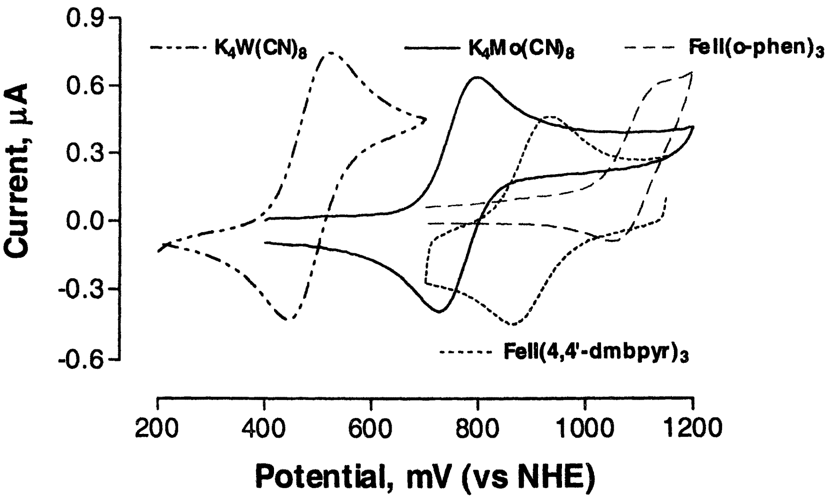

Figure 2. Cyclic voltammograms of transition metal complexes.

We have tested these metal complexes with laccase for their ability to mediate delignification of softwood kraft pulp (20). Results shown in Figure 3, indicate that all four complexes can catalyze laccase delignification of oxygen delignified kraft pulp, and that the best results were obtained with potassium octacyanomolybdate. The rate of electron transfer with the two iron complexes (FeII(4,4'-dmbpyr)$_3$ and FeII(o-phen)$_3$) seems to be too slow to promote a good delignification even though their redox potentials are in the correct range. On the other hand the difference between the redox potentials (0.26V) of potassium octacyanomolybdate versus octacyanotungstate can explain the difference in their efficiencies to delignify pulp.

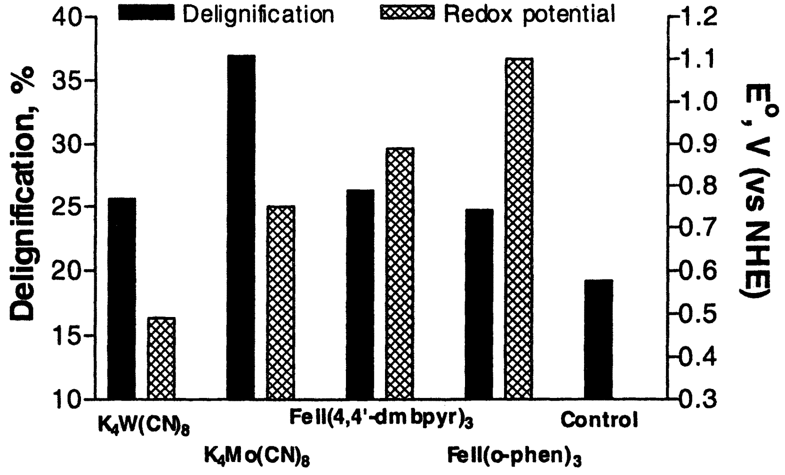

Figure 3. Delignification of softwood oxygen kraft pulp (kappa no= 15) with T. versicolor laccase (10U/g) and transition metal complexes (0.25% on pulp) and followed by a peroxide reinforced alkaline extraction (Ep). The pulp control was treated under the same conditions but without laccase and mediator.

Pulp delignification with recycled octacyanomolybdate

The stability of transition metal complexes and more particularly potassium octacyanomolybdate, was confirmed by measuring, using cyclic voltammetry, the concentration of octacyanomolybdate recovered in the liquor following the

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enzymatic pulp treatment. To take advantage of the stability and reversibility of the mediator, a series of pulp delignification trials were carried out where a fresh solution of mediator (0.1% on pulp) was only added to the first batch of pulp (20). At the end of each batch treatment, the pulp was pressed and the extracted liquor containing the recycled mediator was recuperated and used to treat subsequent batches of pulp. To compensate for loss of enzyme activity, fresh laccase was added at each cycle. Each treatment, including a control where pulp was treated under the same conditions but without laccase and mediator, was followed by an alkaline extraction. As shown in Figure 4, between 33 to 35% delignification were obtained for each batch of pulp as compared to 19% delignification for the control.

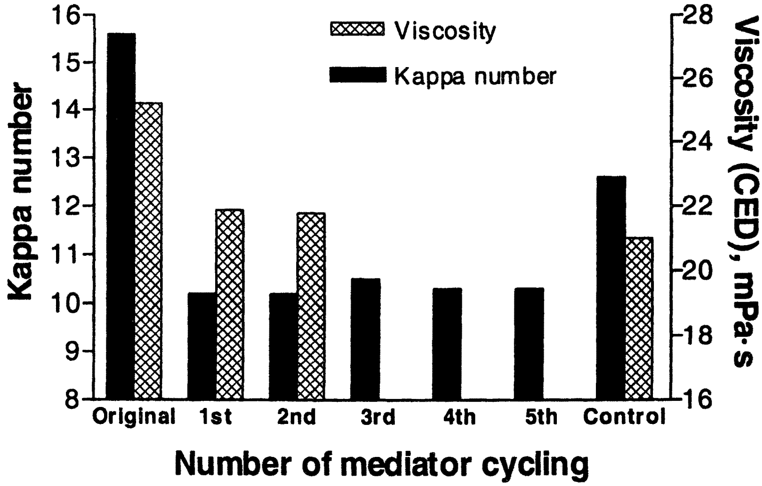

Figure 4. Delignification of softwood oxygen delignified kraft pulp with T. versicolor laccase (10U/g) and recycled mediator ($K_4Mo(CN)_8$; initial charge of 0.1% on pulp). Each treatment was followed by a peroxide reinforced alkaline extraction (Ep)

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Conclusions

The application of laccase in the presence of mediators to oxidize lignin and delignify kraft pulp is now well documented. Several nitrogen containing aromatic compounds have been found to be effective to delignify kraft pulp, but presently none of them are considered cost effective for mill application. A common problem with organic mediators is that due to either their radical intermediate instability or coupling behavior, they cannot perform as true catalysts during delignification. Thus, relatively high initial charges of mediators (around 1%) are required to obtain good performance. Recently, we found that some transition metal complexes in combination with laccase can be used as selective catalytic delignifying agents. Potassium octacyanomolybdate was particularly efficient at low charge with the possibility of being recycled for further cycles of pulp treatment. The principle of action of this new class of mediators is based on their reduced or oxidized states being stable enough to exist in solution under conditions required for pulp treatment. Thus, the transition metal mediator cycles from its reduced state (M^{m+}) to its oxidized state ($M^{(m+1)+}$), following its sequential reaction with laccase and lignin (Figure 5). During this reversible redox process, the mediator is continuously regenerated and made available for further cycles of oxidation by laccase. In this way oxygen is the only component which is consumed during the process.

Experimental

Enzyme and chemicals

Laccase II from *Trametes versicolor* was prepared and its activity measured as described previously (24). ABTS, HBT NNDS, HNNS, promazine, violuric acid and iron tris-o-phenanthroline (Ferroin) were all from commercial sources. NHA was synthesized according to Oxley *et al.* (25). Potassium octacyanomolybdate and potassium octacyanotungstate were prepared

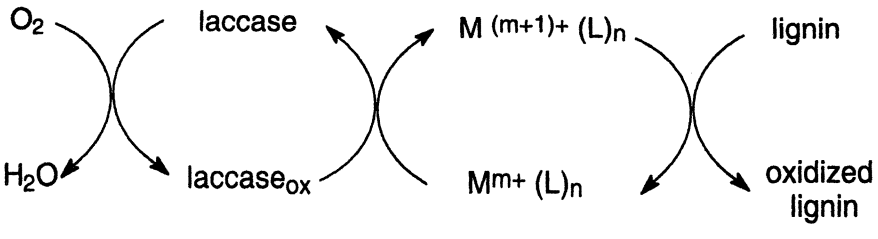

Figure 5. Catalytic cycle of a new class of mediators based on transition metal complexes. M^{m+}: Transition metal ions $(L)_n$: Ligands.

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according to Van de Poel and Neumann (26) and Heintz (27) respectively. FeII(4,4'-dmbpyr)$_3$] was prepared by mixing ferrous chloride with three molar equivalent of 4,4'-dimethyl-2,2'-dipyridyl.

Cyclic voltammetry

Cyclic voltammetry determinations were carried out with a BAS CV-50W Voltammetric Analyser (Bioanalytical Systems Indiana, USA) with an Ag/AgCl reference electrode , a platinum wire auxiliary electrode and a 3mm diameter glassy carbon working electrode. Redox potentials are expressed against normal hydrogen electrode (NHE) by adding 200mV to the potential measured with a silver reference electrode.

Pulp delignification

Acidified softwood oxygen delignified kraft pulp (pH 4 to 5) was mixed in a Hobart mixer for 1 minute with a solution of the mediator and laccase at 10% pulp consistency. The amount of mediator used is specified in % (g per 100 g of oven dried pulp). The pulp suspension was placed in an oxygen pressurized vessel (135 kPa) at 60°C for 2 hours. At the end of the treatment, the pulp was pressed, and the extracted liquor was kept for further batches of pulp delignification (for recycle experiments). After washing with water, the pulp was subjected to an alkaline extraction (2% NaOH) enhanced with hydrogen peroxide (0.28%) (Ep) operated at 70°C for 90 minutes.

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Chapter 25

Development of New Laccase-Mediators for Pulp Bleaching

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Analogs of N-hydroxyacetanilide (NHA), one of the best laccase-mediators reported so far, were prepared and evaluated as laccase-mediators for pulp bleaching. Kinetic properties of laccase for these NHA analogs were also determined. It was found that the kinetic properties of laccase such as K_m and k_{cat} are significantly influenced by the composition of laccase-mediators. It was demonstrated for the first time that more effective laccase-mediators could be developed through chemical modification of another laccase-mediator.

Chlorine and chlorine-based chemicals have conventionally been used for pulp bleaching. The environmental problems associated with chlorine-based bleaching processes are well documented and have led, in part, to the development of the cluster rules(*1*, *2*). The continued growth and success of the pulp and paper industry depends upon technological advances in environmentally benign pulp bleaching processes. Enzymatic pulp bleaching techniques, being environmentally friendly, have drawn much attention in recent years. Xylanases were the first enzymes found capable of saving conventional bleaching chemicals such as chlorine dioxide(*3*) when used commercially in a bleaching sequence. Since the xylanases do not directly degrade lignin, the use of these enzymes has its limitation for pulp bleaching. The research focus has

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ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

therefore been switched to lignin-degrading enzymes such as laccase and manganese peroxidase(*4*).

Laccase alone cannot degrade lignin because of its low redox potential and the inaccessibility of the lignin to direct oxidation by laccase(*4, 5*). However, in the early 1990s, Bourbonnais and Paice discovered that in the presence of a small organic compound, ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate)), (called laccase-mediator or redox mediator), laccase was able to degrade lignin and decrease the pulp kappa number(*6*). Call and Mücke in Germany later discovered another effective laccase-mediator, 1-hydroxybenzotriazole (1-HBT), and applied a laccase/1-HBT system to bleaching of pulps at pilot plant level(*7, 8*). Since then, an intensive race to find a cheaper and more effective laccase-mediator for pulp bleaching has been on. In 1997, Amann reported on several other effective laccase mediators such as N-hydroxyacetanilide (NHA), violuric acid and 3-nitrosoquinolin-2, 4-diol(*8*). A short bleaching time (less than 4 hours) is a prerequisite for a bleaching stage to be used in a pulp mill. With laccase/NHA being able to effectively bleach pulp in a short period of time (2 hrs), a laccase/mediator system (LMS) is believed to be coming closer to commercialization(*9*). However, whether a laccase-mediator can be commercialized in a laccase-based pulp bleaching depends on various factors such as its efficacy, toxicity, cost, and ease of use. An overall evaluation of a laccase-mediator based on these factors has to be made before a LMS can be commercialized. Reports of more laccase-mediators like NHA would certainly allow us to have a better choice of a laccase-mediator for pulp bleaching. In an effort to find a laccase-mediator with superior overall performances for pulp bleaching, we demonstrate here that structural modifications of a laccase-mediator such as NHA can generate laccase-mediators with a stronger pulp bleaching ability than NHA. The chemical features of a laccase-mediator that govern the pulp bleaching effect are also discussed.

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Materials and Methods

Enzyme. Crude *Trametes villosa* laccase (TvL), kindly provided by Novo Nordisk Biotech, Inc., was used in our pulp bleaching studies. TvL isoform-1 (TvL-1) was purified from the crude TvL preparation to apparent homogeneity (one band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels)(*10*). The purified enzyme was used for determination of its kinetic properties, K_m and k_{cat}, with various laccase-mediators as substrates.

Unbleached hardwood pulp. Unbleached hardwood pulp was obtained from a Georgia pulp mill. The pulp was extensively washed with de-ionized water and then stored in a cool room (4°C) before use. Its initial brightness value was 35.0, and the initial kappa number was 13.6.

Laccase-mediators. The following laccase-mediators were obtained from commercial sources: N-Benzoyl-N-phenylhydroxylamine (BPHA), Cupferron (N-nitroso-N-phenylhydroxylamine ammonium salt, NNPH). N-hydroxyacetanilide (NHA) was synthesized according to an established procedure(*11*). The melting point and NMR spectra of the synthesized NHA match the literature data. Syntheses of N-hydroxybutyroylanilide (NHB), N-hydroxy-*tert*-butyroylanilide (NHTB), 2-chloro-N-hydroxyacetanilide (CNHA), and 2-chloro-5-methyl-N-hydroxyacetanilide (CMNHA) were carried out as follows. NMR spectra were recorded at 400 MHz (Bruker 400) at ambient temperature with DMSO-d_6 as the solvent and internal reference (central solvent peak, δ_H, 2.50 ppm; δ_C, 39.5 ppm).

Synthesis of NHB. NHB was synthesized according to a modified procedure for NHA, i.e., by the reduction of nitrobenzene to N-phenylhydroxyamine that was subsequently acylated with n-butyryl chloride(*11*). In a 500-ml, three-necked flask fitted with an addition funnel, condenser and thermometer, a mixture of nitrobenzene (20 g, 163 mmol), tetrahydrofuran (100 ml) and 5% rhodium on carbon (wet, 0.5 g) was magnetically stirred and cooled to 15°C in an ice-water bath. Hydrazine monohydrate was added to the mixture dropwise for 30 min while the reaction temperature was kept at 25-30°C. At the same temperature, the mixture was stirred for 2 more hours and filtered. To the filtrate (the crude N-phenylhydroxyamine) was immediately added slurry of sodium bicarbonate (21 g) and water (20 ml). The mixture was cooled to -4°C in an ice-salt bath. Butyryl chloride (17.64 g, 166 mmol) was added to the well-stirred mixture for 1 hr while the temperature was maintained below 0°C. The ice-salt bath was removed and a solution of sodium hydroxide (10 g) and water (100 ml) was added after the reaction continued for 30 min. The aqueous phase was separated and the tetrahydrofuran phase was diluted with petroleum ether. The aqueous phase was separated and the organic phase was extracted two times with 10% sodium hydroxide solution (25 ml). The aqueous phases were combined, washed with dichloromethane (100 ml), and acidified with cold concentrated hydrochloric acid. The mixture generated was extracted with dichloromethane (3 x 60 ml). The dichloromethane extracts were dried with anhydrous $MgSO_4$, filtered and concentrated at reduced pressure to about 40 ml. NHB crystallized from dichloromethane (21.66 g, 74.4%). δ_H, 0.93 (t, 3H, CH_3); 1.59 (m, 2H, CH_2); 2.54 (t, 2H, CH_2); 7.12-7.63 (m, 5H, aromatic H); 10.46 (s, 1H, NOH); δ_C, 14.0 (CH_3); 17.7 (CH_2); 35.6 (CH_2); 120.6, 124.7, 128.5, 142.1 (aromatic C), 172.3 (C=O). FAB-MS gave an $[M+H]^+$ ion peak at m/z 180 confirming the molecular weight. The FAB-MS spectra also show a strong $[M+H-16]^+$ peak corresponding to the expulsion of a single O atom from the parent molecule, which is characteristic of a HO-N group (*12, 13*).

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Synthesis of NHTB. The procedure for the synthesis of NHTB is basically the same as for NHB, replacing n-butyryl chloride with *tert*-butyryl chloride. NHTB did not crystallized from dichloromethane. The crude NHTB was purified by silica gel chromatography (chloroform then chloroform-ethyl acetate, 19:1) to provide pure NHTB (60%). δ_H, 1.10 (d, 6H, CH_3); 2.44 (m, 1H, CH); 7.13-7.69 (m, 5H, aromatic H); 10.48 (s, 1H, NOH); δ_C, 19.05 (CH_3); 30.93 (CH); 120.99, 124.90, 128.52, 142.23 (aromatic C), 175.99 (C=O). FAB-MS gave an $[M+H]^+$ ion peak at m/z 180 confirming the molecular weight and a strong $[M+H-16]^+$ ion peak confirming the HO-N group(*12, 13*).

Syntheses of CNHA and CMNHA. Syntheses of 2-chloro-N-hydroxyacetanilide (CNHA) (60%) and 2-chloro-5-methyl-N-hydroxyacetanilide (CMNHA) (75%) were performed using the corresponding nitro compounds according to the procedures for the preparation of chloroarylhydroxylamines(*14*) and for the N-acetylation of arylhydroxylamines(*11*) (Values in parentheses are overall yields based on the corresponding nitro compounds). The melting points and NMR spectra of CNHA match the literature data(*15*). FAB-MS gave an $[M+H]^+$ ion peak at m/z 186 for CNHA confirming the molecular weight and a strong $[M+H-16]^+$ ion peak confirming the HO-N group(*12, 13*). NMR spectral data of CMNHA are as follows: δ_H, 2.14 (s, 3H, CH_3); 2.30 (s, 3H, CH_3); 7.18-7.42 (m, 3H, aromatic H); 10.57 (s, 1H, NOH); δ_C, 20.4 (CH_3); 21.1 (CH_3); 128.5, 129.7, 130.1, 130.5, 137.7, 139.2 (aromatic C); 170.5 (C=O). FAB-MS gave an $[M+H]^+$ ion peak at m/z 200 confirming the molecular weight, and a strong $[M+H-16]^+$ ion peak confirming the HO-N group(*12, 13*).

Kinetic properties of TvL with laccase-mediators. The kinetic parameters, K_m and k_{cat}, for TvL with the laccase-mediators were determined using the same procedure as described in our previous paper(*16*).

Enzyme assay. Laccase activity was determined by monitoring the oxidation of ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) (500 μM) at 420 nm (E_{max} = 3.6 X 10^4 $M^{-1}cm^{-1}$). The measurements were made in 50 mM sodium tartrate buffer (pH 4.5) at 30°C. One enzyme unit was defined as 1.0 μmol of product formed per min under the assay conditions.

Determination of bond dissociation energy (BDE) of laccase-mediators. B3LYP/6-31G(d,p) optimizations were done with Gaussian 98, revision A.7(*17*), running on a Cray SV1 supercomputer, administered by the Alabama Supercomputer Authority. Unrestricted calculations were done for the open-shell structures. The calculations were set up through Unichem V5.0, running on a Silicon Graphics Indy Workstation in the Department of Chemistry at Auburn University.

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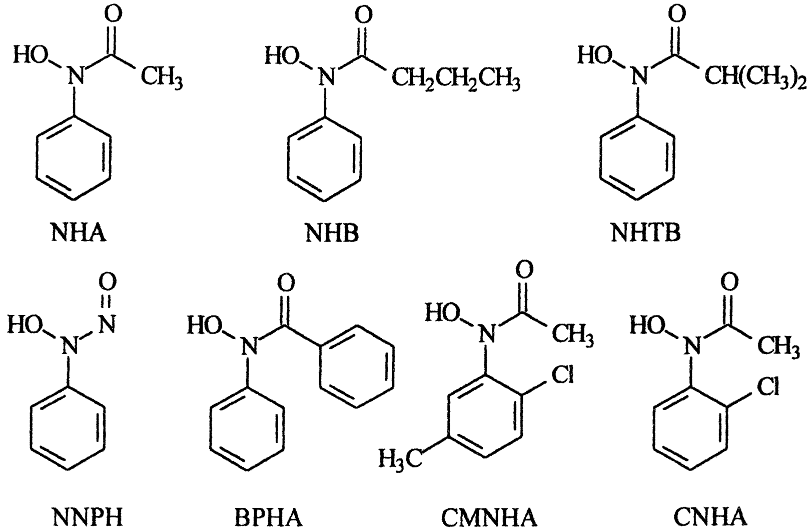
Pulp bleaching procedure with laccase and the derivatives of NHA. The unbleached hardwood kraft pulp (5.0 g, o.d.) was incubated with a mediator (10 mg per gram o.d. pulp) and TvL (100 units per gram o.d. pulp) at 2% consistency in NaOAc buffer (pH=4.5, 50 mM) at 400 kPa of oxygen and at 60°C for 2 hours. The enzyme treated pulp was filtered and washed with de-ionized water. The filtrate was used to measure the residual laccase activity. A half of the enzyme-treated pulp (2.5 g) was extracted with an alkaline solution (2% NaOH on pulp, 10% consistency) at 70°C for 90 minutes. The other half of the enzyme-treated pulp (2.5 g) was bleached with an alkaline H_2O_2 solution (2% NaOH on pulp, 2% H_2O_2 on pulp) for 4 hours at 90°C. Handsheets were formed according to TAPPI test method T218 om-83. Brightness and kappa number of the pulp were measured using TAPPI test method T525 om-86 and the micro kappa number method(*18*), respectively. Brightness and kappa number of the control experiment were obtained from the pulp that was treated with laccase in the absence of a laccase-mediator, and then either extracted by the alkaline solution or bleached with the alkaline H_2O_2 solution as described above.

Results and Discussion

The chemical structures of NHA derivatives are shown in Figure 1. The



NHA NHB NHTB

NNPH BPHA CMNHA CNHA

Figure 1. Chemical structures of NHA derivatives

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structural differences among NHA, NHB, NHTB, NNPH, and BPHA are the substituent on the nitrogen atom. Compared with NHA, CMNHA and CNHA have substituents on the aromatic ring. These compounds, except NNPH, are normally prepared through the reduction of aromatic nitro compounds to their hydroxylamines followed by N-acylation reactions. NHB readily crystallized from dichloromethane and can be prepared as conveniently as NHA. Preparation of NHTB was not as easy as NHA because a purification step with silica gel chromatography was required to obtain pure NHTB. NNPH is commercially available and is the cheapest compound among those shown in Figure 1. BPHA can be either obtained from commercial sources or conveniently prepared in laboratory using the procedure for NHA. Laboratory-scale preparations of CMNHA and CNHA are cheaper and easier than that of NHA because Pd-C, rather than expensive Rh-C, was used as catalyst in the reduction of the aromatic compounds to their hydroxylamines.

Kinetic properties of laccase with these laccase-mediators are shown in Table I. The replacement of the acetyl group in NHA with other carboxylic groups greatly impacts upon the K_m and k_{cat} values of TvL for these compounds (Table I).

Table I. Kinetic properties of *T. villosa* laccase with N-hydroxy compounds[a] and residual laccase activity after the pulp bleaching with a laccase/mediator system.

Mediator	*K_m(mM)*	*k_{cat} (min^{-1})*	*BDE (kcal/mol)*	*Residual laccase activity (U/ml)[b]*
NNPH	1.4 ± 0.9	590 ± 80	83.333	0.645±0.012
NHA	0.9± 0.3	476±60	84.588	0.714±0.042
NHB	3.5 ± 0.5	140 ± 6	85.090	0.722±0.025
NHTB	14 ± 4	300 ± 30	84.776	0.702±0.03
BPHA	0.4 ± 0.2	81 ± 7	83.145	0.661±0.029
CNHA	8.2±3.3	140±16	84.714	0.858±0.011
CMNHA	7.5 ± 1.6	89 ± 6	84.588	0.927±0.018

[a] The K_m/k_{cat} values are results of nonlinear regression fitting of Michaelis-Menten equation on 6 to 10 experiment readings that covered the whole rate-[substrate] profile (from initial, linear phase to the saturated phase).

[b] Data are the means of three independent experiments.

Theoretically, the bond dissociation energy (BDE) of RO-H bond would have linear relationship with log(k_{cat}/K_m) (k_{cat}/K_m is the approximate second-order rate constant of the oxidation of laccase substrate) when other factors such as substrate binding do not affect the oxidation. Through studies of the oxidation of a series of phenols, anilines and benzenethiols by fungal laccases, Xu indeed observed a linear relationship between log(k_{cat}/K_m) and redox potential difference(ΔE) between laccase and substrate, although significant deviation exists for some substrates with bulky substituents around the phenolic hydroxy group ($\Delta E \propto$ BDE)(*19*). A poor linear relationship (R^2=0.3798) between log(k_{cat}/K_m)s of these NHA analogs and their BDEs is shown in Figure 2 (The unit of $k_{cat}/K_m$is $M^{-1}min^{-1}$). NHTB has the highest K_m probably because the bulky *tert*-butyryl group hinders the binding between NHTB with laccase active site. CMNHA and CNHA have the next highest K_ms (Table I). It has been demonstrated that the N-OH group and the phenyl group are orthogonal to each other, i.e. the OH group does not reside in the plane of the aromatic ring(*15, 20*), which could account for the high K_ms of laccase for CMNHA and CNHA. The lowest K_m value for BPHA could be, in part, due to its planar structure and its π system(*21*). As a result, the substrate specificity of laccase may not be as low as we have previously thought, especially for those non-conventional laccase substrates (Phenols are considered as conventional substrates for laccase).

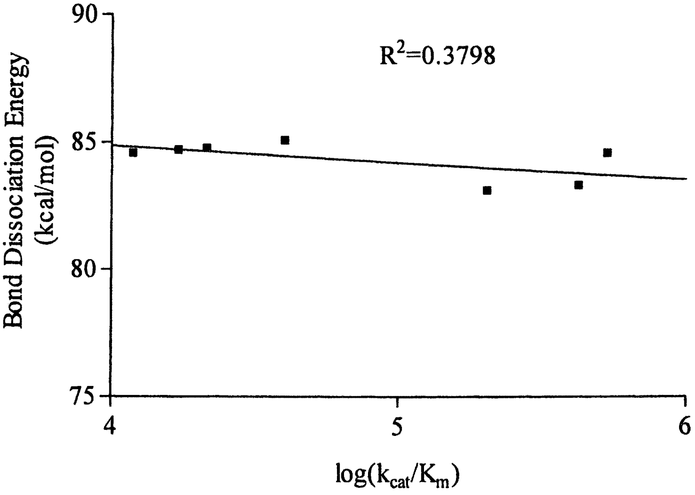

Figure 2. Relationship between BDEs and log (k_{cat}/K_m)

Treatment of the unbleached kraft pulp with laccase and NHA derivatives followed by an alkaline extraction all resulted in equivalent or higher brightness than that obtained with NHA (Figure 3). More importantly, the derivatives resulted in lower (or equivalent) kappa numbers than that obtained with NHA (Figure 4), indicating their abilities to degrade lignin. It has been demonstrated that inactivation of laccase is one of the factors that affect the

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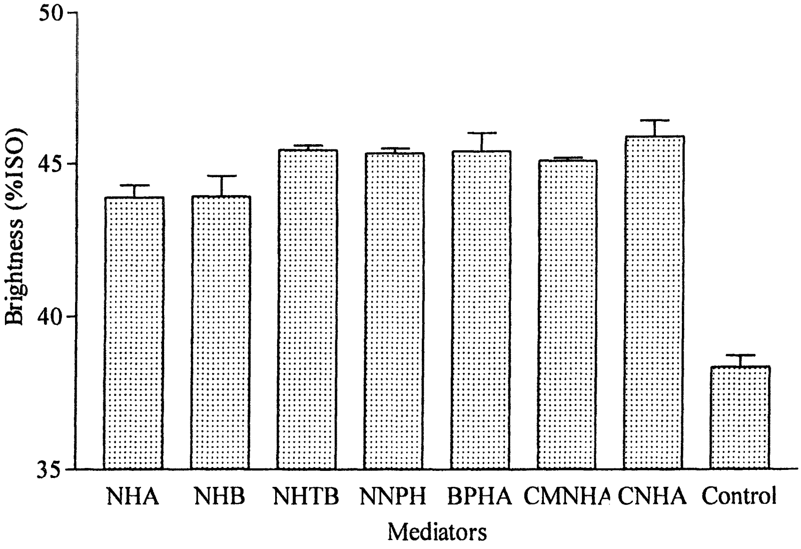

Figure 3. *Brightness (%ISO) after an alkaline extraction. Error bars show the SD of data from three independent experiments.*

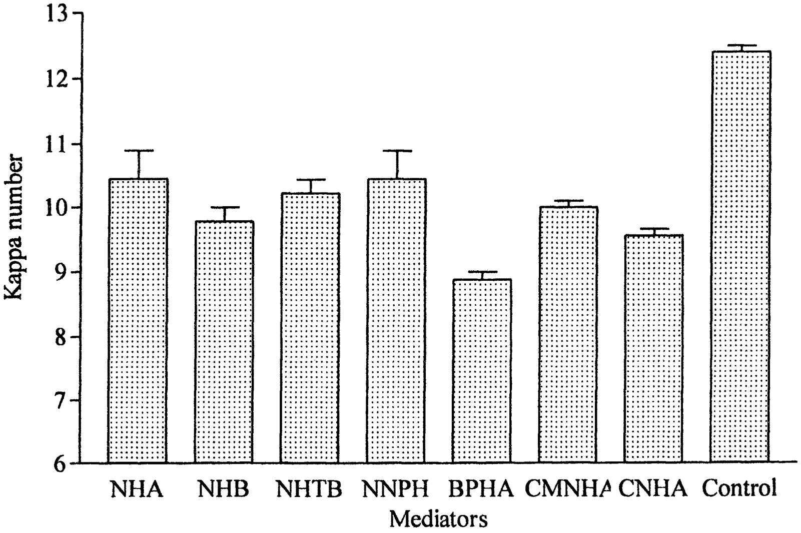

Figure 4. *Kappa number after an alkaline extraction. Error bars show the SD of data from three independent experiments.*

efficacy of a LMS for pulp bleaching (especially when 1-hydroxybenzotriazole was used as a laccase-mediator)(*9, 22*). In an effort to compare the contribution of laccase-mediators for pulp bleaching, a relatively high dosage of laccase was used in this study. As we can see, the residual laccase activities after the incubation of the pulp with laccase and the NHA derivatives are basically the same for all of these mediators (Table I).

A simplified scheme for laccase-based pulp bleaching is shown in Figure 5. Pulp bleaching by a laccase/mediator system is dependent upon factors as follows: 1) the ability of laccase to oxidize a laccase-mediator as indicated by K_m and k_{cat}; 2) properties of the free radical of the laccase-mediator, including its stability and redox potential. In comparing fungal laccases and laccase-mediators (1-hydroxybenzotriazole and violuric acid) in the oxidation of a non-phenolic lignin model compounds, we demonstrated that the oxidation rate of the non-phenolic lignin model compound by a LMS depends on both k_{cat} and the inactivation of laccase(*16*). With the residual laccase activities almost the same, the increase in the brightness (Figure 3) and the decrease in the kappa number (Figure 4) are not correlated with k_{cat} or k_{cat}/K_m (Table 1), which implies that oxidation of lignin by the free radicals of NHA derivatives is the rate-limiting step in the delignification of the pulp.

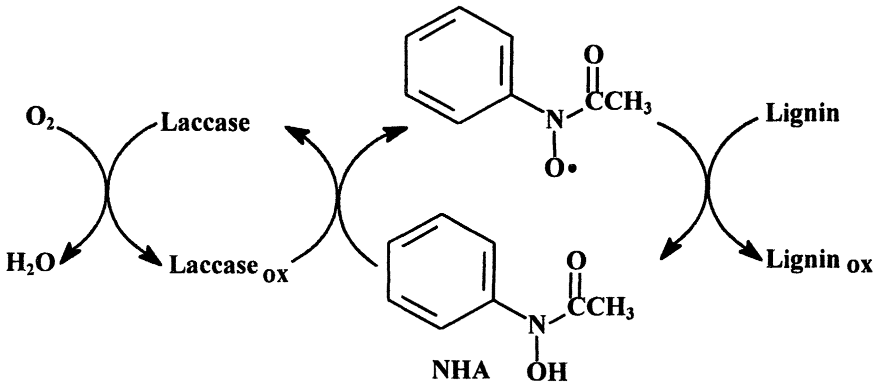

Figure 5. A simplified scheme for the oxidation of lignin by laccase/NHA

The NO• free radical from NHA is believed to be the actual oxidants for the degradation of lignin(*23*). In pulp bleaching, the NO• free radical from an effective mediator should have long enough lifetime for it to penetrate the pulp fiber and oxidize the lignin. The importance of the stability of the oxidized mediator in pulp bleaching has been discussed previously(*23, 24*). Stability of an NO• free radical is generally correlated with the BDE of the NO-H bond, i.e.,

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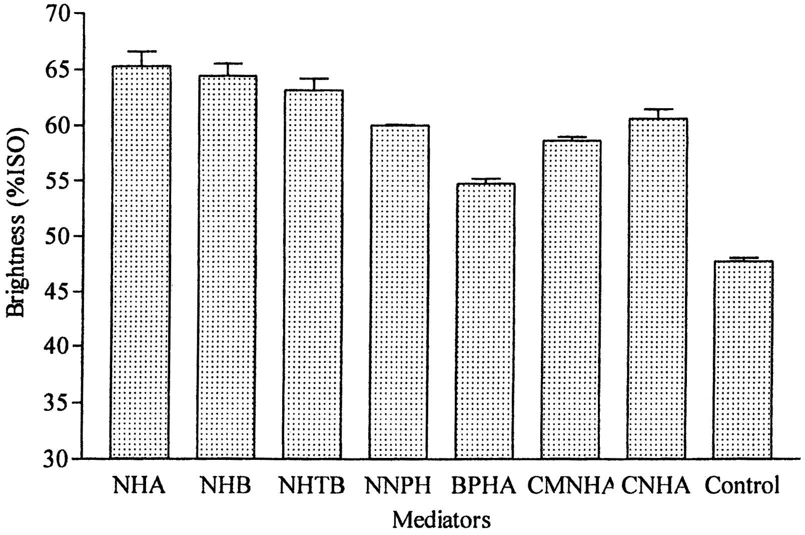

Figure 6. *Brightness (%ISO) after an alkaline H_2O_2 bleaching. Error bars show the SD of data from three independent experiments.*

the lower the BDE the more stable the free radical. In this context, the low BDE of a laccase-mediator is good for pulp bleaching by a LMS. However, the weak O-H bond strength of NHA derivatives generally resulted in the slow hydrogen abstraction from an organic substrate such as lignin by the NO• free radical(*25*), which implies that a low BDE of a laccase-mediator has a negative effect on pulp bleaching by a LMS. A typical example to demonstrate the relationship of stability of a free radical and its reactivity is 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO). The low O-H BDE of TEMPOH (69.6 kcal/mol) makes TEMPO free radical very stable, but very inert for hydrogen abstraction as well(*26*). Studies on the electrochemical oxidation of seven N-hydroxy-N-arylacetamides (RNOHCOCH3, R=phenyl, 4-chlorophenyl, 3,4-dichlorophenyl, 4-ethoxyphenyl, 2-fluorenyl and 2-phenanthryl) by cyclovoltammetry have revealed two different oxidation potentials, a quasi-reversible one-electron transfer in the range between 0.55 and 0.63 V and an irreversible transfer of a second electron between 0.80 and 1.20 V, for each of these compounds (*27*). The decay rates of aryl NO• free radicals were highly dependent upon the aryl groups(*27*). It is unclear whether oxidation of NHA derivatives by laccase would also result in two types of oxidations. Further investigation is warranted to quantitatively correlate the properties of NO• free radicals of NHA derivatives with their ability to degrade lignin in pulp.

Compared with the enzymatic treatment of pulp followed by an alkaline

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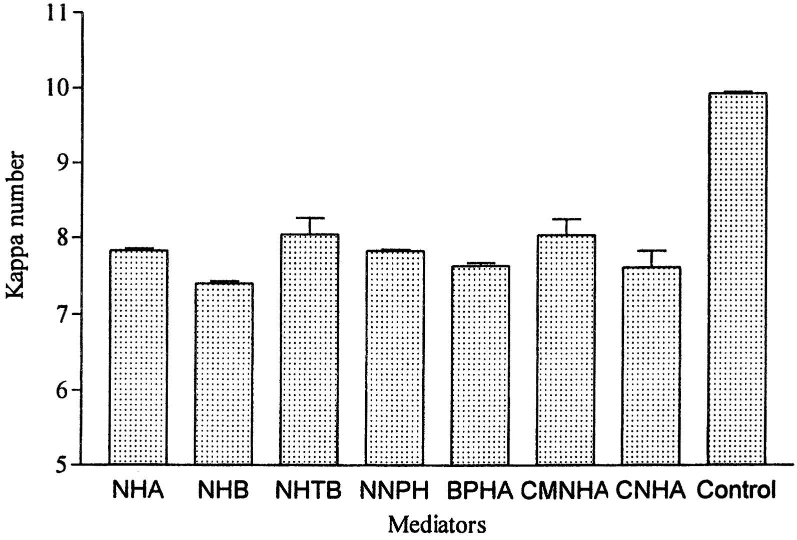

Figure 7. *Kappa number after an alkaline H_2O_2 bleaching. Error bars show the SD of data from three independent experiments.*

extraction, a post-treatment with an alkaline H_2O_2 solution resulted in higher brightness and lower kappa number (Figures 6 and 7). From figure 6, we can see that NHA has the highest brightness while the BPHA has the lowest. However, NHA has the similar kappa number with other mediators (Figure 7). In conventional pulp bleaching, the increase in pulp brightness generally accompanies with the decrease in pulp kappa number. The discrepancy between brightness increase and kappa number decrease in these laccase-based pulp bleaches is associated with the absorbance of a laccase-mediator or its degradation products on the pulp. Degradation of a laccase-mediator could form chromophores, thereby lowering pulp brightness. A typical example is ABTS, the first laccase-mediator used for pulp bleaching. Pulp would become colored (pulp brightness decreases) after treated with laccase/ABTS although lignin has been significantly degraded (*6*). Moreover, hydrogen peroxide could be partly consumed by the mediator or its degradation products in the post-treatment, thereby resulting in lower brightness or higher kappa number. Quantitative determination of a laccase-mediator and its degradation products that absorb on the pulp is difficult to accomplish because of the complex nature of its degradation products. The measurement of the remaining mediator in the solution of pulp slurry through HPLC indeed indicated that laccase-mediators have different binding capacities on unbleached pulp with the concentration of BPHA in the solution being the lowest among these compounds (data not shown).

NNPH is especially noteworthy here. NNPH, commercially available as an ammonium salt, is very soluble in water. All compounds, except NNPH, have to be dissolved in an organic solvent such as dimethylformamide before adding to pulp. High solubility of a laccase-mediator in water is one of the important features when a LMS is used for pulp bleaching. From figures 3-4 and 6-7, we can see that NNPH is as effective as NHA in pulp bleaching regardless of the post-treatments. This demonstrates that a highly water-soluble laccase-mediator could be as effective as NHA, one of the best laccase-mediators, for laccase-based pulp bleaching.

In summary, efficacy of a mediator for pulp bleaching can be enhanced through the modification of its chemical structure. Oxidation of lignin by the free radical generated from NHA derivatives appeared to be the rate-limiting step. Reports of a variety of effective laccase-mediators would allow us to have a better choice of a mediator when commercializing a LMS for pulp bleaching.

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Chapter 26

Expression of Laccase I and IV Genes from *Trametes versicolor* in *Trichoderma reesei*

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The laccase I and IV genes from *Trametes versicolor* were subcloned into a fungus specific expression vector comprising the promoter and secretion signal of *Trichoderma reesei cbh1* gene and transcription terminator of the *cbh2* gene with a *pyr4* selection marker for growth on minimal media. This vector was introduced by biolistic bombardment into a proprietary production strain of *Trichoderma reesei.* Southern hybridization permitted identification of gene copy number in transgenic strains. The transgenic fungi successfully expressed laccase in shake flask cultures as measured by reaction of ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonicacid)) with laccase at 30 °C. The Cu^{2+} concentration in the growth medium was optimized in 14 L pilot fermentations. Under optimized conditions the best laccase IV transgenic strain produced 800-1000 mg/L of laccase.

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Introduction

Laccase enzymes have been studied for three decades and their utility for a wide number of applications has been recognized (1,2,3,4,5). The laccase applications which have been demonstrated thus far are primarily in the fields of pulp delignification and bleaching, oxidation of dyes, effluent treatment, deinking of recycled paper and the polymerization of aromatic compounds in effluent, particularly in lignin containing effluents from pulp bleaching or cellulose hydrolysis. For some applications a redox active mediator compound is required and while development of suitable mediators is underway (6,7) the costs associated with the custom synthesis of novel mediator compounds are high. To develop a cost effective laccase mediator system it is necessary for the enzyme production cost to be a minor component of the total cost of the system. Since quantities of enzyme produced by wild type laccase producing strains are low, heterologous expression and scale up fermentation of recombinant laccase is a desirable approach towards increasing production of laccase enzymes and lowering overall enzyme costs. A number of investigations have produced recombinant laccase enzyme from a variety of different production strains using laccase genes from different fungal species. These include genes from *Polyporus pinsutus, Trametes villosa, Myceliophthera thermophilia, Scytalidium thermophilium, Coprinus cinerus, Neurospora crassa, Trametes versicolor, Schizophyllum commune* and *Phlebia radiata.* The expression hosts investigated include a number of industrially significant microorganisms such as *Aspergillus sp., Neurospora crassa, Pichia pastoris, Saccharomyces cerevesiae* and *Trichoderma reesei* (8, 9, 11, 12, 13, 14). These publications report yields of laccase protein in the range of 20-149 mg/L.

Of particular significance for performing pulp bleaching is the redox potential of laccase enzymes. In only a few cases have the redox potentials of laccase enzymes been determined (8,14,15) and the redox potentials of *Trametes* laccases have been reported to be the highest (15). The aim of this study was to achieve expression of high redox potential laccase in a commercially viable expression system for a variety of industrial uses. For this reason *Trametes* laccase genes were selected for expression in proprietary industrial strains of *Trichoderma reesei.* We aimed also to construct recombinant *Trichoderma* strains producing laccase at levels 10 times that of the native *Trametes versicolor.*

Materials and Methods

Strains and Plasmids

Trametes versicolor strain 52J (ATCC # 96186) and plasmids pBK116 and pBK117 containing the *T. versicolor* laccase I and laccase IV cDNA clones (16) were provided by Fred Archibald, PAPRICAN. *Trichoderma reesei* strain BTR213, a proprietary strain of Iogen Corporation, is a low protease derivative of RutC30 (ATCC # 56765).

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Vector Construction

PCR amplification of laccase genes was conducted using primers designed from published sequence information (16) and using the plasmids pBK116 and pBK117 as templates. The forward primer *gctctagagccattgggccc* contained an *Xba* I restriction site (underlined) and reverse primer *ccgctcgagtcagaggtcgga* contained an *Xho* I restriction site (underlined). Amplification was carried out under the following condition: 95 °C for 5 min, 38 °C for 2 min, 72 °C for 2 min, followed by 26 cycles of 95 °C for 2 min, 38 °C for 2 min, 72 °C for 2 min with a 10 minute final extension at 72 °C. PCR products were run on 1 % agarose gel. The 1.6 and 1.7 kb laccase bands were excised, purified and ligated into the EcoRV site of pBR322 to produce pB41 and pB11 and transformed into XL1 Blue *E.coli* cells. Restriction digestion of these plasmids with *Xba* I and *Xho* I generated fragments with suitable ends for subcloning into pCHE2cor provided by Iogen Corp. Ottawa, to produce the expression vector pCLaccIV and pCLaccI. pCHE2cor consisted of the *cbh*1 promoter and secretion signal and the *cbh*2 terminator. This vector was further digested with *Xba* I and *Not* I to produce a 5 kb fragment that ligated to the *pyr*4 selection marker producing pCLaccI-TV or pCLaccIV-TV (Figure I).

Transformation of *Trichoderma reesei*

The Biolistic PDS-1000/He system (BioRad; E.I. DuPont de Nemours and Company) was used to transform spores of *T. reesei* strains BTR213 and all procedures were performed as recommended by the manufacturer. Gold particles (median diameter of 0.6 um) were used as microcarriers. The

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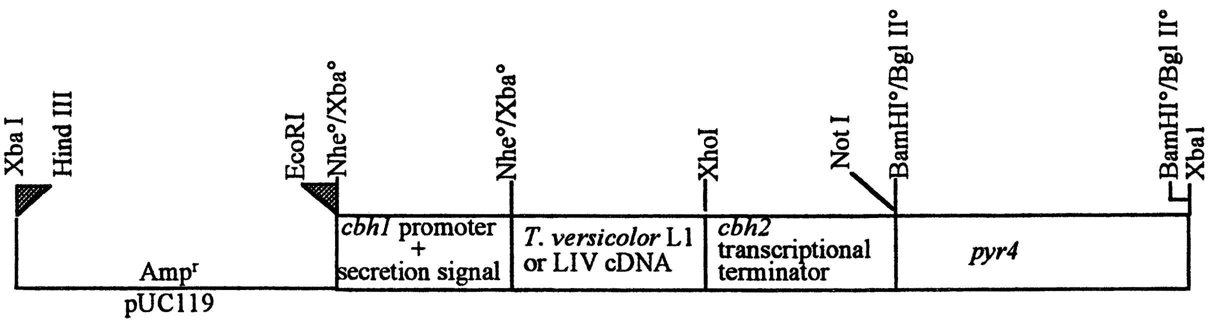
Xba I
Hind III
EcoRI
Nhe°/Xba°
Nhe°/Xba°
XhoI
Not I
BamHI°/Bgl II°
BamHI°/Bgl II°
XbaI
Amp^r
pUC119
cbh1 promoter + secretion signal
T. versicolor L1 or LIV cDNA
cbh2 transcriptional terminator
pyr4

Figure I: Vectors for the expression of T. versicolor laccase I and IV in T. reesei. The coding sequences of the laccase *I* and laccase *IV* cDNA clones were operably linked to the promoter and secretion signal of the *T. reesei cbh1* gene. The plasmids were linearized at the unique *Xba* I site prior to transformation of a uridine auxotroph of *T. reesi* strain BTR213. The shaded triangles represent the remaining restriction sites present in the pUC119 polylinker (*XbaI-SalI-PstI-SphI-HindIII* and *XbaI-BamHI-SmaI-KpnI-SacI-EcoRI*)

following parameters were used in the optimization of the transformation: a rupture pressure of 1100 psi, a helium pressure of 29 mm Hg, a gap distance of 0.95 cm, a macrocarrier travel distance of 16 mm, and a target distance of 9 cm. Plates were prepared with 1×10^6 spores on Minimal Media Agar (MMA). Bombarded plates were incubated at 28°C. Transformants were observed after 3-6 days growth. Further incubation was necessary to achieve sporulation.

Minimal Media Agar

Minimal Medium consisted of: Glucose 10 g/L, KH_2PO_4 10 g/L, $(NH_4)_2SO_4$ 6 g/L, $MgSO_4.7H_2O$ 1 g/L, Tri-sodium citrate 3 g/L, $FeSO_4.7H_2O$ 0.005 g/L, $MnSO_4.H_2O$ 0.0016 g/L, $ZnSO_4.7H_2O$ 0.0014 g/L, $CaCl_2.2H_2O$ 0.002 g/L. Bacto agar 15 g/L, pH 5.5, 1ml /L Trace Elements where the trace elements consisted of: $FeSO_4.7H_2O$ 5 g/L, $MnSO_4.H_2O$ 1.6 g/L, $ZnSO_4.7H_2O$ 1.4 g/L.

Screening for Laccase Producing Transformants

Transgenic fungi were picked onto fresh minimal media agar plates containing 1 mM $CuS0_4$, 0.5 mM ABTS and 1 % Solka Floc. Green halos were observed around positive transformants expressing laccase activity after 48 hr.

Shake Flasks and Fermentation

Individual colonies of *Trichoderma* were transferred to PDA plates for the propagation of each culture. Sporulation was necessary before inoculation of shake flasks which were used in determining the yield of laccase produced by a culture. The culture media was composed of the following: $(NH_4)_2SO_4$ 6.3 g/L, KH_2PO_4 4.0 g/L, $MgSO_4$-$7H_2O$ 2.0 g/L, $CaCl_2.2H_2O$ 0.5 g/L, Corn Steep Liquor 6.2 g/L, $CaCO_3$ 10.0 g/L, Glucose 5.0 g/L, Solka Floc 10.0 g/L, Trace elements 1 mL/L. Trace elements solution contained $FeSO_4.7H_2$ 05.0 g/L; $MnSO_4.H_20$ 1.6 g/L; $ZnSO_4.7H_20$ 1.4 g/L. The carbon source was sterilized separately as an aqueous solution at pH 2-7 and added to the remaining media. The liquid volume per 1 liter flask was 150 mL, the initial pH was 5.5 and each flask was sterilized by steam autoclave for 30 minutes at 121°C prior to inoculation. Spores were isolated from the PDA plates and enumerated. 2×10^6 spores were used to inoculate each flask. Flasks were shaken at 200 rpm at a temperature of 28°C for a period of 6 days. The filtrate containing the secreted protein was collected by filtration through GFA glass microfibre filters (Whatman). Growth in a 14-liter fermentation vessel was carried out using the

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procedures described above with twice the media concentration levels listed, and without Solka Floc. *Trametes versicolor* strain 52J was grown in 14-Liter fermentations on mycological broth at pH 5.0 at 28 °C for 5-7 days.

Protein Determination

The protein concentration was determined using Bio-Rad Protein Assay (Cat. No. 500-0001) using *Trichoderma* cellulase as a standard.

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Laccase Activity

ABTS oxidation (0.5 mM final concentration) was performed in 0.1 M sodium acetate pH 5.0 at 30 °C monitoring the change at A420 with an extinction coefficient of 3.6×10^4 M^{-1} cm^{-1} to calculate the rate of oxidation and laccase units per milliliter (LU/ml).

Determination of Cellulase Activity

Cellulase activity was determined by measuring the release of reducing sugars from filter paper using the method of Ghose (10).

DNA preparation

Genomic DNA from *Trichoderma* strains was isolated from biomass collected from Potato Dextrose Broth (Difco) grown cultures shaken at 200 rpm for 2-3 days at 28°C. The mycelia was filtered onto a GFA glass microfibre filter (Whatman). The fungal cakes were frozen in liquid nitrogen and crushed into a powdered biomass. 0.1 g were resuspended per ml of TE, pH 7.5 plus 1% sodium dodecyl sulphate (SDS). The lysate was centrifuged and was extracted with of saturated phenol followed by extraction buffer-saturated phenol:chloroform:isoamyl alcohol (25:24:1). DNA was precipitated from the solution by adding 3M sodium acetate, pH 5.2 and cold 95% ethanol. The DNA was pelleted by centrifugation, rinsed with 70% ethanol, air-dried and resuspended in TE, pH 8.0. RNA was digested by the addition of 0.1 mg/ml Ribonuclease A (Boehringer Mannheim) and incubation at 37°C for 1 hour. The RNAse was removed by additional extraction with phenol/chloroform. The final DNA pellet was resuspended in TE, pH 8.0. The concentration of DNA was determined by measuring the absorbance of the solution at 260 nm.

Southern Blots

For Southern blots, genomic DNA was isolated from each strain as described. One μg of DNA was digested with 3-10 units of *Xho* I restriction endonuclease at 37°C for at least 2 hours and the digestion products resolved on a 0.8% agarose gel in 0.04 M Tris-acetate, 1 mM EDTA. DNA was transferred to nylon membranes (Boehringer Mannheim) by capillary transfer. Since *Xho* I cuts once in the integrated vector, a laccase probe will detect fragments from the integrated vector that terminate at the internal *Xho* I site and an *Xho* I site within the host genome. Therefore the number of hybridizing bands greater than 3.3 kb equals the number of full length copies. Southern blots were hybridized with a digoxigen-11-dUTP labeled random-primed probes prepared using the DIG Labeling and Detection Kit (Boehringer Mannheim). The template for the probes used were the 1.7 and 1.6 kb fragments comprising laccase I and IV mature coding region. After post-hybridization washes, dig-dUTP complexes were visualized by incubation with an anti-digoxigenin alkaline phosphatase conjugate (Boehringer Mannheim) followed by reaction with 5-bromo-4-chloro-3-indoyl phosphate and 4-nitro blue tetrazolium chloride (Boehringer Mannheim).

Results

Vectors for laccase expression in *Trichoderma* (Figure I) were constructed and introduced into *Trichoderma reesei*. Twenty percent of laccase IV transformants and ninety percent of laccase I transformants were found to be positive for laccase expression as determined by the formation of green halos around the colonies on plates containing ABTS. Transformed strains grown in shake flasks produced 0.05 - 0.1 mg/ml of protein. Table I shows gene copy number of the laccase I and IV genes in each positive laccase I and IV transformant and the corresponding LU/ml produced in shake flasks. Multiple gene copies in a transformant did in some cases (1303b, 1303c, 1303d) increase the yield of laccase over a single gene copy. The highest laccase yield was however produced by strain (1156d) which contained only a single laccase IV gene copy. To further enhance laccase production optimization of copper supplements to each individual transformant was carried out (Figure II).

The optimal copper supplement required for maximum laccase production was identified to be characteristic for each individual transformant. Using this information, 14 L scale fermentation was carried out under optimized

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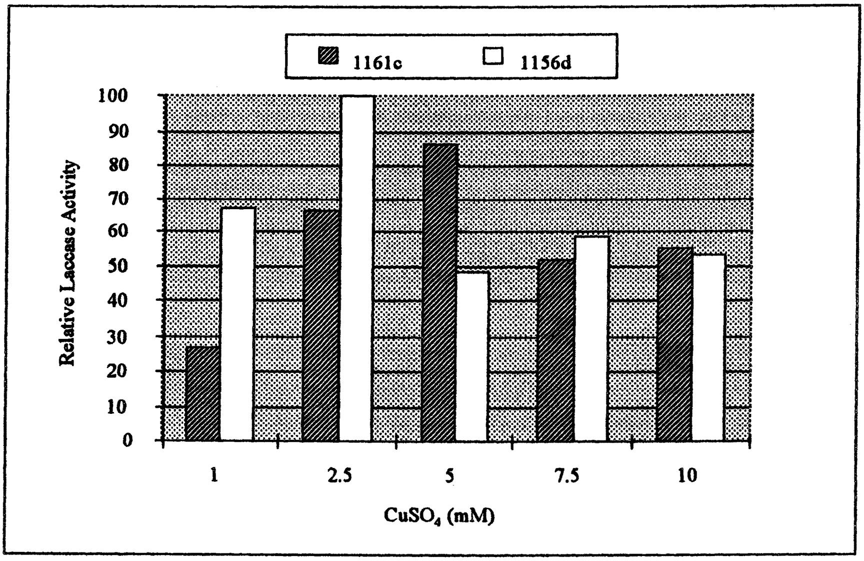
Table I: Laccase gene copy number and laccase activity of Laccase I and Laccase IV transformants grown in shake flasks

Laccase I			Laccase IV		
Strain	Copy #	LU/ml	Strain	Copy #	LU/ml
1301a	1	0.12	1156d	1	2.4
1301c	1	0.01	1156e	1	0.4
1302d	3	0.07	1157c	4	1.0
1303b	3	0.39	1157e	1	0.3
1303c	3	0.28	1157x	2	0.1
1303d	3	0.26	1158a	2	0.7
1304a	1	0.12	1158d	2	ND
1304b	1	0.12	1159c	2	0.8
1304c	1	0.12	1161c	1	0.4
1304d	1	0.10	1167a	1	0.4
T. reesei	0	0.00	*T. reesei*	0	0.0

Figure II: Laccase production by two T. reesei transformants over a range of different copper concentrations in shake flasks



conditions for the best laccase I and IV transformants. These results are shown in Table II.

Table II: Laccase, cellulase and protein production by laccase transformants in 14 L scale fermentation

Strain	Laccase gene	Copper	Laccase Activity	Specific Activity	Protein	Cellulase Activity
		(mM)	LU/ml	LU/mg	mg/ml	FPU/mg
T. versicolor	Multiple	0.5	7.5	18.75	0.4	ND*
1161c	Lacc IV	1.0	108	47.3	2.28	1.65
1156d	Lacc IV	1.0	230	31.2	7.37	1.12
1304b	Lacc I	1.0	1.5	2.0	0.75	0.05
1303b	Lacc I	0.5	1.15	0.67	1.17	ND*
T. reesei	None	1.0	ND**	ND**	23.2	1.39

ND**- Not detected, ND* - Not determined

The best laccase IV transformant, 1156d, produced 230 LU/ml which is significantly more laccase activity (LU /ml) than both the best laccase I transformant 1304b (200 fold) and the native *Trametes versicolor* (25 fold). Since the purified laccase IV protein from strain 1156d has a specific activity of 200 LU/mg (Table III), the yield of laccase IV protein is about 1.0 g/L. However SDS gels indicate the purified laccase IV is approximately 80 percent pure (Figure III - Lane 6)), so the yield may be as low as 800 mg/L which is still significantly higher than any previously reported laccase yields.

The expression of laccase in *Trichoderma* reduced the overall protein production: 3-10 fold in the case of laccase IV and 25 fold in the case of laccase I. The very low cellulase activity (FPU/mg) in the fermentation broth of laccase I transformants showed that expression of laccase I caused a severe reduction in cellulase production, whereas laccase IV expression in *Trichoderma* did not significantly affect cellulase production. In addition, SDS-PAGE analysis of the fermentation broths showed a significant changes in the relative proportions of the cellulase components produced by the recombinant strains as compared to the parent strain (Figure III). Unlike the fermentation broth from the parent strain, cellobiohydrolase I is no longer the most abundant protein in the broths from the recombinant strains. This is particularly evident in the broth of the laccase I strain 1304b, which is consistent with the significantly reduced filter paper activity (Table II). In addition, the broths from the recombinant strains contain little or no endoglucanase 3.

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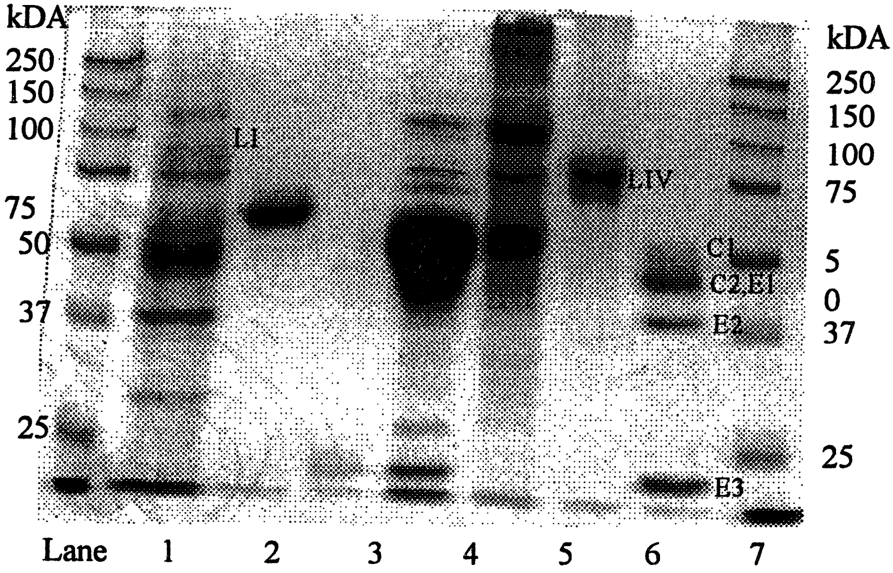
Figure III: SDS Gel of recombinant laccase proteins in Trichoderma reesei



Lane 1 - 10 µg crude fermentation broth of 1304b (Laccase I), Lane 2 -10 µg purified *Trametes versicolor* Laccase II, Lane 4 - 10 µg curde fermentation broth of BTR213, Lane 5 - 10 µg crude fermentation broth of 1156d (Laccase IV), Lane 6 - 10 µg of purified recombinant Laccase Ifrom strain 1156d, Lane 7 - purified *Trichoderma* cellulase components (cellobiohydrolases C1 and C2, endoglucanases E1, E2 and E3) total of 4 µg loaded.

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Table III: Comparison of native and recombinant laccase I and recombinant laccase IV

Sample	Observed MW (kDA)	Calculated MW (kDa)	Specific Activity (LU/mg)
Fermentation broth 1156d (Laccase IV)			17.4
Recombinant Lacc IV (Purified)*	77	54	200.0
Fermentation broth 1304b (Laccase I)			0.5
Recombinant Lacc I (Purified*□)	90	54	26.1
LI and II Fermentation Broth			5.2
Native Lacc I (Purified)**	60**	54	12.1

* Purified using S-Sepharose ion exchange column, eluted with a stepwise gradient in sodium acetate pH 5.0 upto 0.2 M,

□ Chromatofocussed on PBE94 with Polybuffer 74 pH 3.0

** Purified according to (17)

SDS-PAGE analysis also indicates the appearance of novel proteins of 90 kD and 77kD produced by the laccase I and IV transformants, respectively, that are not produced by the parent strain (Figure III, lanes 1, 4 and 5). The 77kD band present in the fermentation broth of the laccase IV strain comigrates with the purified laccase protein from that same broth. For both recombinant laccase I and IV, the recombinant enzymes are of higher molecular weight than the native *Trametes* laccase I enzyme (67 kD--reference 17) and the purified *Trametes* laccase II enzyme (60 kD--Figure III, lane2). This indicates that the recombinant laccase enzymes produced in *Trichoderma* are more extensively glycosylated than the native *Trametes* laccases. Based on the differences in the observed and calculated molecular weights, glycosylation accounts for 30-40% of the weight of the recombinant enzymes and only 10-20% of the weight of the native enzymes.

Discussion

The results of this study report the successful heterologous gene expression of laccase in *Trichoderma reesei* using the expression vector comprising of the *cbh1* promoter and secretion signal and *cbh2* terminator. The yield of laccase IV protein achieved is 6-7 times higher than previously reported highest yields for heterologous laccase expression which was produced in *Aspergillus oryzae*

(9). The only report of laccase expression in *Trichoderma reesei* (11) details the heterologous expression of the laccase gene from *Phlebia radiata* using an expression construct containing the *cbh1* promoter and terminator, and the native *Phlebia* laccase secretion signal. Under these conditions, 20 mg/L of laccase was produced in contrast with the 800-1000 mg/L reported in this study. It is surprising that the use of a native secretion signal and *cbh1* terminator in the expression construct should limit the yield of laccase to such an extent. However there are a number of other factors that may impact on expression and protein yields. Most clearly host strain and location of integration of the expression vector into the genome may be responsible. For example the high level of laccase IV by strain 1156d from only a single copy of the expression vector indicates that the site of integration in this strain may be a more actively transcribed locus.

The identity of the laccase gene expressed can also have a significant effect on yield of laccase activity. The same expression construct containing laccase I or IV produced significantly different yields. At this stage it is unclear what accounts for this. Northern blots of total RNA isolated from transgenic *Trichoderma* suggest that laccase mRNA transcript levels are similar in both laccase I and IV strains and that this is unlikely to be responsible for the difference (data not shown). In addition, laccase protein secretion is accompanied by a decrease in overall protein secretion and changes in cellulase expression by the cells (Table II. Figure III). Further studies aim to investigate whether laccase protein has remained inside the cells and blocked secretion pathways or whether the laccase protein has damaged the secretion apparatus of the cell.

This study has also identified that individual transgenic strains have different copper requirements which can specifically affect laccase protein production. Saloheimo and Niko-Paovola (11) suggest that because the specific activity of the native and the *T. reesei* produced *Phlebia* laccases were similar, all of the laccase prosthetic groups are accurately incorporated into the apo-laccase produced by *T. reesei*. However this does not discount the possibility that under limiting copper conditions, apo-laccase may be degraded either intra or extracellularly and that the additional copper present in the media used in the experiments presented prevents this degradation of apo-laccase from occurring.

Comparisons of native and recombinant laccase show that for the purified recombinant laccase I not only was there a higher specific activity than the native enzyme (Table III) but preliminary kinetic characterization indicates it also has 10 fold higher Km and Vmax values for ABTS oxidation. The reasons for this are currently under investigation and may include the presence of other

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ABTS oxidizing enzymes in the native laccase I preparation. The interference of the more extensive glycosylation of the recombinant enzyme, as evidenced by the higher observed molecular weight (Table III), with the binding and release of ABTS and / or its oxidation product may also be responsible.

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Chapter 27

Evaluation of the Delignification and Bleaching Abilities of Selected Laccases with HBT on Different Pulps

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South Africa

The laccases of *Peniophora sp.*, *Pycnoporus sanguineus*, *Trametes versicolor* and *Trametes hirsuta* have been used in the presence of a mediator, 1-hydroxy-benzotriazole (HBT), to delignify and bleach various pulps. The effects induced by the laccase/HBT-treatment on pulp brightness, kappa number and viscosity were examined. It was found that delignification improved when increased enzyme and mediator dosages were used. The extent of kappa number reduction was dependent on the pulp type and enzyme source. The pulps were accessible to delignification in the following descending order: unbleached hardwood sulfite pulp>post-oxygen softwood kraft pulp>unbleached softwood kraft pulp>post-oxygen hardwood soda pulp. It was demonstrated that biobleaching with *T. hirsuta* laccase/HBT using a DED sequence could increase pulp brightness by 2.9-12.2 points, alternatively, savings of chlorine dioxide of 40-60% could be obtained. On the other hand, the pulp viscosity was influenced by the selectivity of the particular laccase/HBT system used.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Introduction

The growing public sensitivity towards the negative environmental impacts of the chlorine-based pulp bleaching has focused research on the development of new environmentally friendly technologies. Among them biobleaching using enzymes has shown great potential in minimizing the use of chlorine-containing bleaching chemicals. In the last decade xylanases have been extensively investigated and already industrially applied (1). The use of oxidative enzymes such as laccases in pulp bleaching is still on laboratory or pilot plant scale (2).

Laccases belong to the multicopper oxidases (1.10.3.2) which can reduce elemental oxygen to water in a four-electron step and simultaneously perform a one-electron oxidation of many aromatic substrates (3). Due to the redox potential, laccase alone can only oxidize phenolic lignin structures. The addition of a mediator, a small chemical compound, extends the substrate range to non-phenolic lignin structures (4-7). It is assumed that the mediator is needed because the large laccase molecule can not enter the secondary cell wall and oxidize lignin directly.

The first compound described to function as a mediator was 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sufonic acid) (ABTS) (3,8). Laccase did not improve the bleachability of pulp when acting alone (9) although direct oxidation of the lignin has been reported (10). The use of ABTS and laccase demethylates and delignifies kraft pulp (11). Another compound used as a mediator was 1-hydroxybenzotriazole (HBT). HBT is more effective on pulp than ABTS (12) although HBT forms benzotriazole which is inactive as mediator (5,13).

The laccase/HBT treatment of pulp was reported to increase the content of carbonyl and carboxyl groups and reduce the amount of free phenolic hydroxyl and methoxyl groups of residual lignin (14-15). Additional changes in the physical and optical properties of pulp also occurred (16). Over 50% delignification of softwood kraft pulp with *Polyporus* laccase/HBT was achieved (13). Using *T. hirsuta* laccase/HBT, 43% delignification of pine kraft pulp and 60% delignification of post-oxygen pine kraft pulp was reported (17). However, both ABTS and HBT are not available as bulk chemicals and are not completely environmentally safe.

Considerable efforts have been invested in the screening for more efficient and environmentally friendly mediators (18-19). Although a number of compounds have been identified as possible mediators, one which fulfills all the requirements has not yet been identified. The extent of pulp delignification with laccase-mediator systems (LMS) was found to be dependent on both the type of mediator and laccase source. For instance, using laccase preparations from six

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different fungal strains with HBT and ABTS, pine kraft pulp was delignified between 19% and 40% (20).

In this work, four microbial laccases in conjunction with HBT have been studied to evaluate their ability to delignify and bleach different pulps. In an effort to reveal the bleaching power of the LMS, the potential brightness gains and savings of chlorine dioxide using a DED bleaching sequence have been determined.

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Materials and Methods

Enzyme Preparations

Prior to use, the culture supernatants of *Peniophora sp.*, *Trametes versicolor* and *Pycnoporus sanguineus*, grown on 4% molasses for 14 days, were subjected to a 40-fold concentration using an Amicon ultrafiltration cell (10 kDa molecular mass cut-off). The laccase of *Trametes hirsuta* was kindly provided by Dr. Matti Siika-aho from the VTT Biotechnology and Food Research, Finland. Its production and purification has been described previously (14).

Enzyme Assays

The laccase activities were determined using 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sufonic acid) (ABTS) as substrate (9). One unit of enzyme activity was defined as that amount of enzyme that can release 1 μmol of ABTS oxidized per minute. The cellulase activity was determined according to the DNS method using carboxymethyl cellulose as substrate (21). All enzyme preparations were cellulase-free.

Pulps

The industrial pulps used in this work were supplied from various Sappi mills: unbleached softwood kraft pulp; post-oxygen softwood kraft pulp; unbleached hardwood sulfite pulp and post-oxygen hardwood soda pulp. The pulps were thoroughly washed with distilled water until a neutral pH of the wash waters was attained and then used in the experiments without any prior pretreatment or preparation. Kappa number and brightness of these pulps are shown in Table I.

Enzyme/HBT Treatment (L) and Alkaline Extraction (E) of Pulps

Washed pulps (3 g dry weight) were treated with laccase/HBT (L-treatment) at enzyme charges of 5, 10 and 15 U/g pulp (dry weight) and pH 4.5 (*T. versicolor, P. sanguineus* and *T. hirsuta* laccases) and 5.0 (*Peniophora* laccase). The mediator dosage was 2% and 4% (on unbleached kraft pulp), 1% and 2% (on post-oxygen kraft pulp), 0.5% and 1% (on both unbleached sulfite and post-oxygen soda pulps). The pulp treatments were carried out at 55°C and 10% pulp consistency for 3 h under 3.5 bar O_2 pressure. Thereafter, samples of the reaction mixture were withdrawn for determination of the residual laccase activity using the ABTS assay. Pulps were then extracted with 0.7% NaOH (on pulp) at 67°C and 10% consistency for 67 min (E-treatment), washed and dried. Brightness, kappa number and viscosity were determined. Alkali-extracted pulps (E-pulps) and DED-bleached pulps, omitting the L-treatment, served as controls in the delignification (LE) and biobleaching (LE-DED) experiments, respectively (Table I). All experiments were carried out in triplicate.

Table I. Properties of pulps used as controls

	Unbleached kraft pulp		*Post-oxygen kraft pulp*		*Unbleached sulfite pulp*		*Post-oxygen soda pulp*	
Treatment	*Kappa number*	*Brightness(%)*	*Kappa Number*	*Brightness (%)*	*Kappa number*	*Brightness (%)*	*Kappa Number*	*Brightness (%)*
None	22.2	20.4	10.8	29.8	5.2	57.0	4.5	64.2
E	21.6	21.3	10.4	30.9	5.1	57.0	4.4	64.9
DED	ND	ND	ND	62.2	ND	91.6	ND	88.6

NOTE: ND, Not determined; E, Alkaline extraction; D, Chlorine dioxide.

Analysis of Pulp Properties

Kappa number of the LE-treated pulps was determined according to TAPPI Test Method T236 cm-85. Pulps from the individual experiments were pooled together for kappa number determinations of unbleached sulfite pulp and post-oxygen soda pulp. Viscosity of the LE-treated and bleached pulps was determined according to Tappi Test Method T230 om-89. Brightness of pulp handsheets was measured with an Elrepho 2000 instrument based on Tappi Test

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Method T452 om-92. Methanol release from pulp was examined in the enzyme filtrates using a HP 5890 gas chromatograph.

Chlorine Dioxide Bleaching of LE-treated Pulp

Post-oxygen kraft pulp, unbleached sulfite pulp and post-oxygen soda pulp (5 g dry weight) were treated with the laccase of *T. hirsuta* and *P. sanguineus* at a charge of 15 U/g and 2% HBT (on post-oxygen kraft pulp) or 1% HBT (on the other two pulps). The LE-treatment was followed by a D_1ED_2 bleaching performed under the following conditions:

- D_1-step: 2.63% active chlorine on pulp (or reduced as indicated); 128 min; 67°C; 10% consistency
- E-step: 0.7% NaOH on pulp; 82 min; 67°C; 10% consistency
- D_2-step: 1.315% active chlorine on pulp (or reduced as indicated); 195 min; 67°C; 10% consistency

Results and Discussion

Residual Laccase Activity

The residual laccase activity was determined after completion of the L-treatments to ensure that enough enzyme was added to successfully perform the enzymatic reactions over the entire incubation period. It was found that the amount of residual laccase activity depended on the enzyme origin, pulp type, laccase/mediator dosages used in the experiments. These activities as obtained with 1% HBT varied between 3% and 100% of the initial activity (Figure 1). Between 65% and 100% of the *P. sanguineus* laccase was still active after treatment. The highest residual activity was determined on post-oxygen kraft pulp, followed by unbleached sulfite pulp and post-oxygen soda pulp. The use of greater mediator and enzyme charges resulted in lower residual laccase activities (data not shown). Only trace amounts of methanol were detectable in the enzyme filtrates (data not shown).

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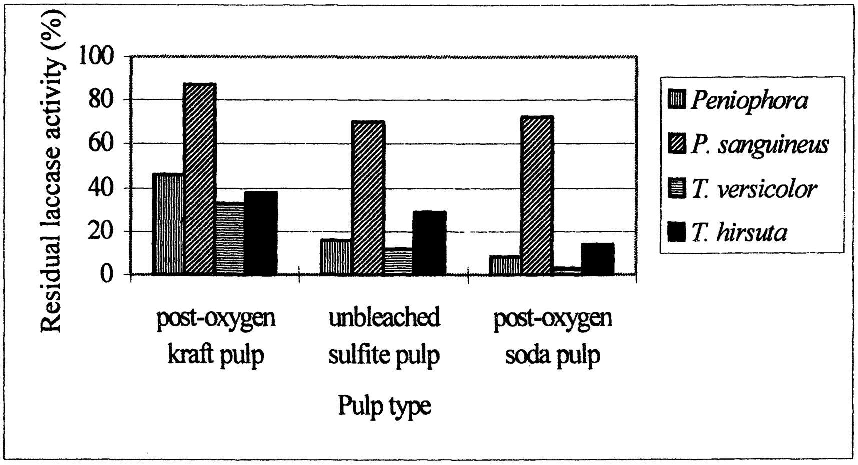

Figure 1: Impact of pulp type on residual laccase activity following L-treatment with 15U/g and 1% HBT

LE-treatment of Pulps

Unbleached Kraft Pulp

The delignifying effect of the LE-treatment on unbleached kraft pulp, expressed as kappa number reduction over the E-treated unbleached kraft pulp, revealed notable differences related to the treatment conditions used (Figure 2). A further delignification of pulp was achieved by increasing the enzyme and mediator dosages. For instance, the kappa number reductions attained using the highest enzyme (15 U/g) and mediator dosage (4%) were 18.3% (*Peniophora* laccase), 15.7% (*P. sanguineus* laccase), 21.3% (*T. versicolor* laccase) and 19% (*T. hirsuta* laccase).

The LE-treatment induced no brightness gains on unbleached kraft pulp (Figure 3). The brightness change was negative in all experiments except when 5 U/g of *Peniophora* laccase and 2 % HBT were used. The brightness loss was more pronounced when increased enzyme and mediator dosages were used. A brightness decrease of up to 2.7 points was obtained with 15 U/g of *P. sanguineus* laccase and 4% HBT. Apparently the darkening effect observed is due to chromophore changes in pulp which occur during the laccase/HBT treatment.

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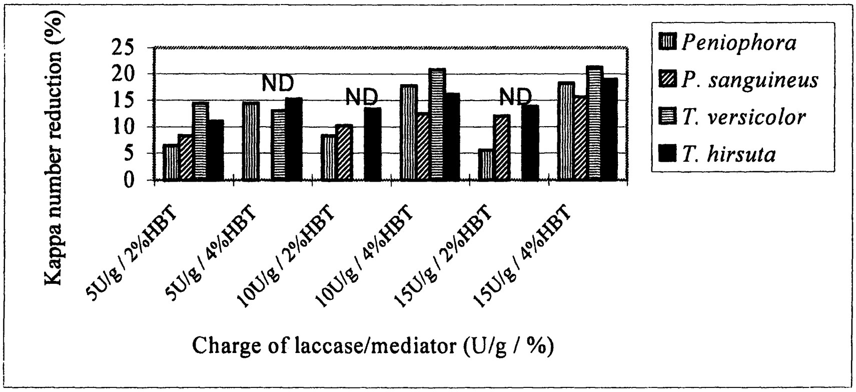

Figure 2: Impact of LE treatment on kappa number of unbleached kraft pulp

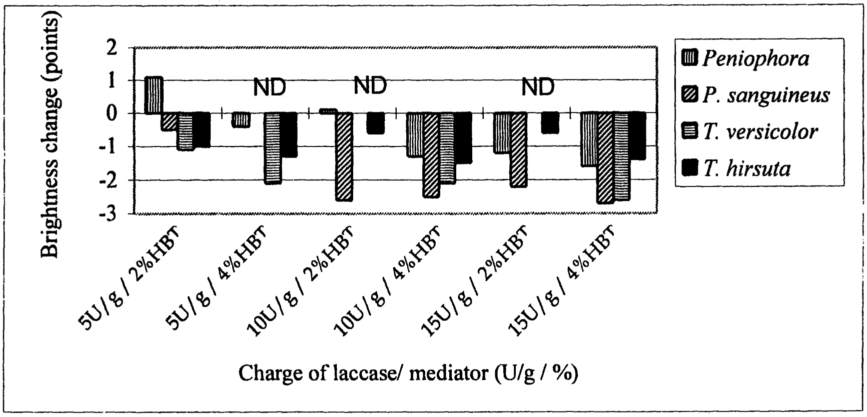

Figure 3: Impact of LE treatment on brightness of unbleached kraft pulp

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Post-oxygen Kraft Pulp

The L-treatment of post-oxygen kraft pulp was carried out with 1% and 2% HBT so to keep a ratio between kappa number and mediator dose similar to that used in the experiments with unbleached kraft pulp. Compared to unbleached kraft pulp, however, the effect of the LE-treatment on post-oxygen kraft pulp was more profound, as shown in Figure 4. Treatment of post-oxygen kraft pulp with the *Peniophora, T. hirsuta* and *P. sanguineus* laccases resulted in kappa number reductions similar to those observed on unbleached kraft pulp. However, the *T. versicolor* laccase was able to decrease kappa number by 19.2% (5 U/g and 1% HBT) as compared to 14.4% (5 U/g and 2% HBT) obtained on unbleached kraft pulp. Apparently, the rate of delignification could be improved by increasing the enzyme and mediator dosage as judged by the results obtained on all pulps with 15 U/g and 2% HBT. Treatment with the two *Trametes* laccases brought about the greatest reductions in kappa number: 29% with *T. versicolor* laccase (10 and 15 U/g with 2% HBT and 15 U/g with 1% HBT) and 26% with *T. hirsuta* laccase (15 U/g and 2% HBT).

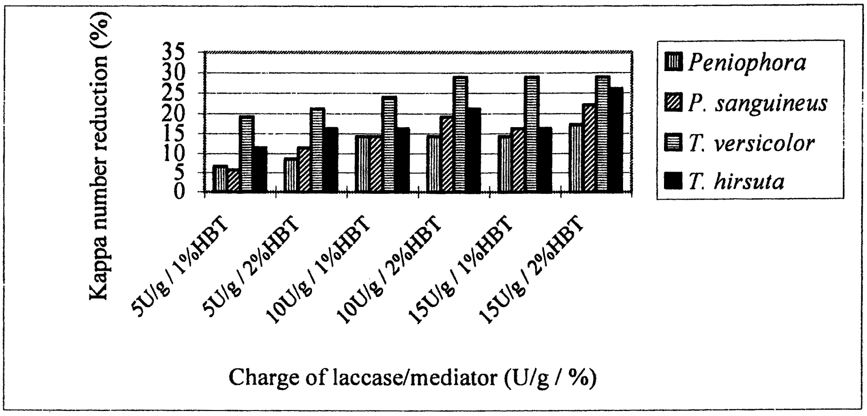

Figure 4: Impact of LE treatment on kappa number of post-oxygen kraft pulp

In most instances, in contrast to unbleached kraft pulp, post-oxygen kraft pulp gained brightness following LE-treatment (Figure 5). The enzymes induced different brightness effects on pulp. While *T. hirsuta* and *T. versicolor* laccases increased brightness by up to 2.3 points (with 15 U/g and 2% HBT), however, the *P. sanguineus* enzyme in fact darkened the pulp by 1.1 points (15 U/g and 1% HBT) and 0.6 points (15 U/g and 2 % HBT). On the other hand, the laccase of *Peniophora* could not cause any substantial change in brightness under the conditions used (Figure 5). Because of the poor performance of this enzyme, results obtained on the other pulps are not shown.

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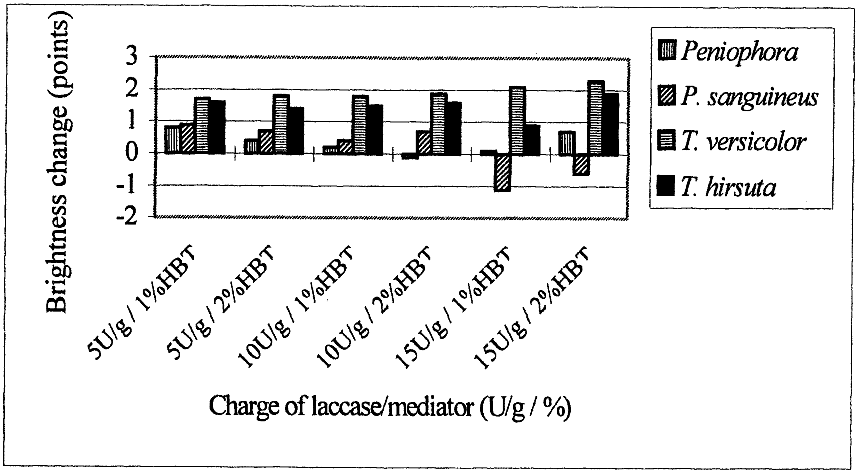

Figure 5: Impact of LE-treatment on brightness of post-oxygen kraft pulp

Unbleached Sulfite Pulp

The LE-treatment of unbleached sulfite pulp was carried out using mediator dosages of 0.5% and 1% on pulp. As shown in Figure 6, best results were obtained with the two *Trametes* enzymes: 51% with *T. versicolor* (10 U/g and 1% HBT) and 49% with *T. hirsuta* (10 U/g and 0.5% HBT). In both instances, any further increase in the enzyme and HBT charges did not improve delignification. Treatment of unbleached sulfite pulp with the laccase of *P. sanguineus* resulted in lower kappa number reductions (up to 15.7%) as compared to the *Trametes* laccases. While the laccases from *Trametes* brightened the pulp by up to 3.4 points (Figure 7), the LE-treatment of unbleached sulfite pulp with the *P. sanguineus* laccase resulted in significant brightness losses (up to 6 points). Treatment of unbleached sulfite pulp with *Peniophora* laccase reduced kappa number less than 9% and decreased pulp brightness by 3 points (data not shown).

Post-oxygen Soda Pulp

The LE-treatments of post-oxygen soda pulp with the laccases of *P. sanguineus, T. versicolor* and *T. hirsuta* (0.5 % and 1.0 % HBT) resulted in kappa number reductions in the range of 9 % to 22 % (Figure 8). These results were more comparable to that obtained on unbleached kraft pulp (Figure 2) than

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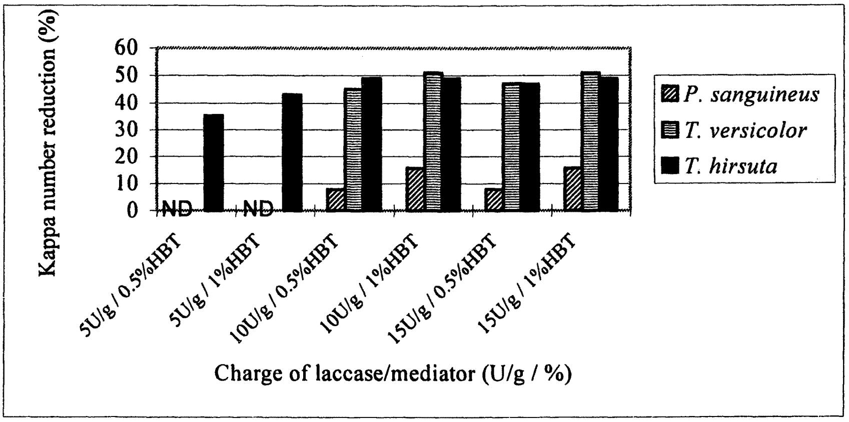

Figure 6: Impact of LE-treatment on kappa number of unbleached sulfite pulp

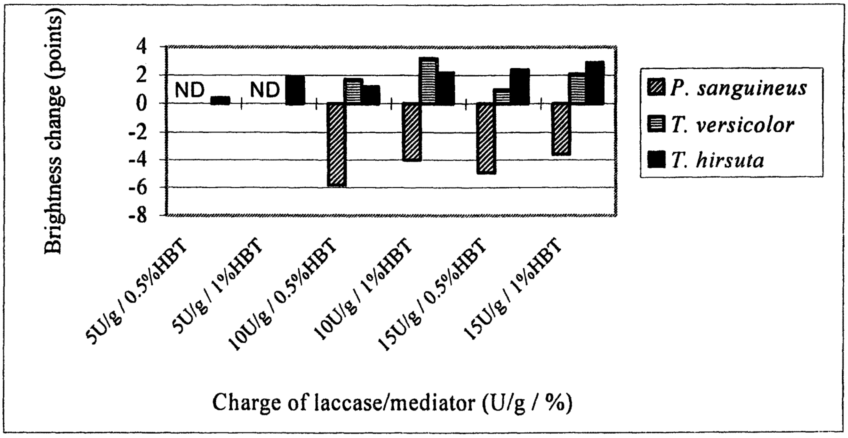

Figure 7: Impact of LE-treatment on brightness of unbleached sulfite pulp

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unbleached sulfite pulp. As shown in Figure 8, the *T. hirsuta* laccase was the best performing enzyme with kappa number reductions of 22.7% (10 U/g and 0.5% HBT). The increase in either HBT or enzyme dosage did not improve delignification. The laccase of *P. sanguineus* reduced kappa number by 11.4% (Figure 8). The *Peniophora* laccase effected only 6.8% reduction of kappa number and had no impact on brightness (data not shown).

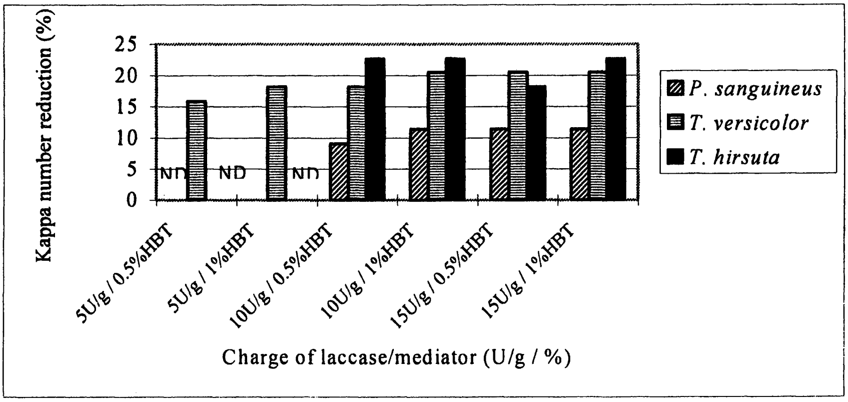

Figure 8: Impact of LE-treatment on kappa number of post-oxygen soda pulp

As shown in Figure 9, the LE-treatment of post-oxygen soda pulp produced significant brightness gains. Thus, the *P. sanguineus* laccase increased brightness by up to 5.1 points (15 U/g and 1% HBT) whereas *T. versicolor* and *T. hirsuta* brightened pulp by 8.5 points (15 U/g and 0.5% HBT) and 9.4 points (10 U/g and 1% HBT), respectively.

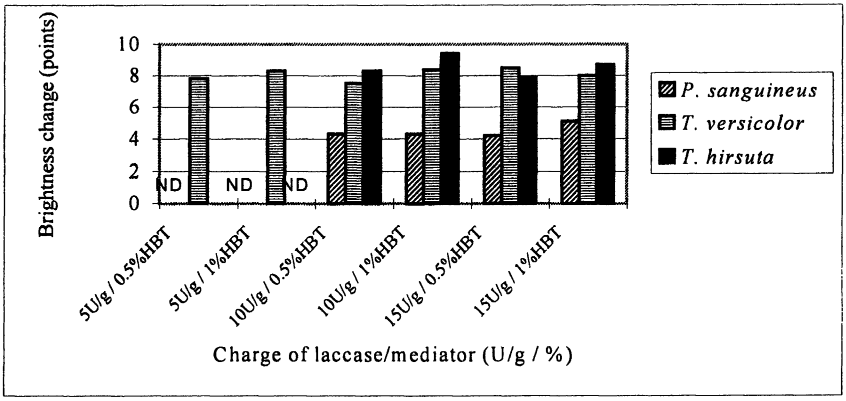

Figure 9: Impact of LE-treatment on brightness of post-oxygen soda pulp

Chlorine Dioxide Bleaching of LE-treated Pulps

An ECF bleaching of the LE-treated pulps was carried out in order to reveal the bleaching potential of the laccase/HBT systems and to estimate the possible savings in chlorine dioxide. All three pulps (unbleached kraft pulp was not used in the bleaching experiments due to its weak response to the LE-treatments) were bleached in a D_1ED_2 beaching sequence using full or reduced (20-60%) charges of chlorine dioxide.

As already shown, the two *Trametes* enzymes performed very similarly during the delignification experiments. The LE-treatments with *T. versicolor* laccase/HBT resulted in slightly greater kappa number reductions on post-oxygen kraft pulp and unbleached sulfite pulp compared to *T. hirsuta* laccase. However, the latter was more efficient in boosting the brightness of unbleached sulfite pulp and post-oxygen soda pulp. The laccase of *P. sanguineus* was included in the bleaching experiments for comparative purposes: to compare its action with that of a *Trametes* type laccase as well as to determine if the different LE- treatment effects produced by the two enzymes would be translated and carried forward in the full LE-DED bleaching.

Post-oxygen Kraft Pulp

Bleaching of *T. hirsuta* laccase-pretreated post-oxygen kraft pulp using the full chlorine dioxide charges effected a brightness gain of 12.2 points over the DED-bleached control (Figure 10). The pulp, bleached with 40% less chlorine dioxide, was 0.7 points brighter than the control. The laccase of *P. sanguineus,* which showed previously weaker delignification abilities than *T. hirsuta* laccase, also induced a less pronounced brightness effect. Nethertheless, using a full chlorine dioxide charge, bleached pulp, which was 7.0 points brighter than the control, was produced. However, a 30% reduction of the chlorine dioxide dose led to a decrease of 2.6 points in the final brightness. Using a *T. hirsuta* laccase/HBT system, brightness of unbleached pine kraft pulp was improved by 2.4 points in a TCF bleaching whereas a brightness gain of 8 points was obtained on post-oxygen pine kraft pulp (17).

Unbleached Sulfite Pulp

The differences in the abilities of the two enzymes to delignify unbleached sulfite pulp (15.7% kappa number reduction with *P. sanguineus* laccase and 49.0% with *T. hirsuta,* respectively) were also reflected in the biobleaching experiments. As shown in Figure 11, treatment of unbleached sulfite pulp with *T. hirsuta* laccase improved brightness by 2.9 points. The DED bleaching using 60% less chlorine dioxide produced pulp only 0.2 points darker than the control. The *P. sanguineus* enzyme could not improve brightness even when full chlorine dioxide charges were used and the reduction of the chlorine dioxide doses resulted in negative brightness changes.

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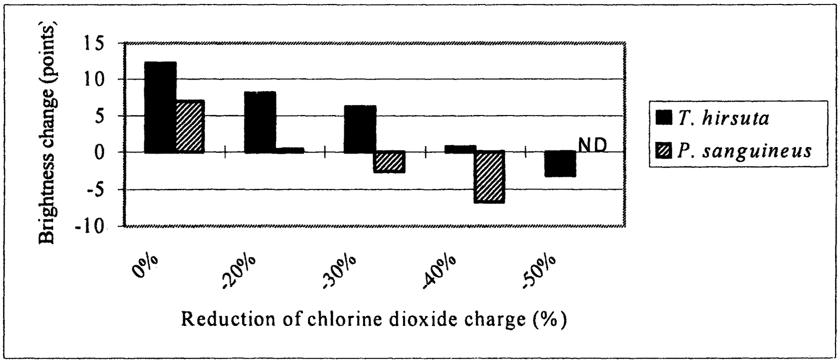

Figure 10: Impact of LE- pretreatment on reduction of ClO_2 charges used in D_1ED_2 bleaching of post-oxygen kraft pulp

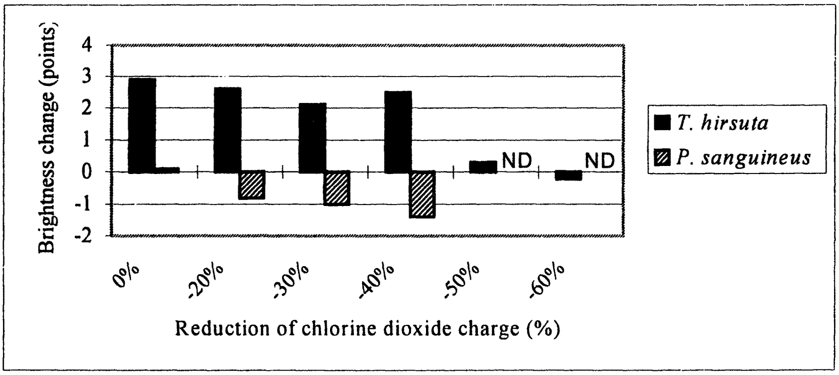

Figure 11: Impact of LE- pretreatment on reduction of ClO_2 charges used in D_1ED_2 bleaching of unbleached sulfite pulp

Post-oxygen Soda Pulp

Biobleaching of post-oxygen soda pulp with the *T. hirsuta* and *P. sanguineus* laccases improved the final brightness by 2.9 and 1.9 points, respectively (Figure 12). This is a surprising result since the two enzymes were very efficient in the LE-treatments of post-oxygen soda pulp (Figure 9) where the corresponding brightness gains were 8.7 points and 5.1 points, respectively. Although *P. sanguineus* laccase reduced kappa number by only 11.4% after LE, a 40% reduction in the D-charge was still possible after LE-DED thereby the brightness of the resulting pulp was still higher than the control. On the other hand, biobleaching with *T. hirsuta* laccase indicated that savings of chlorine dioxide greater than 60 % seem to be possible.

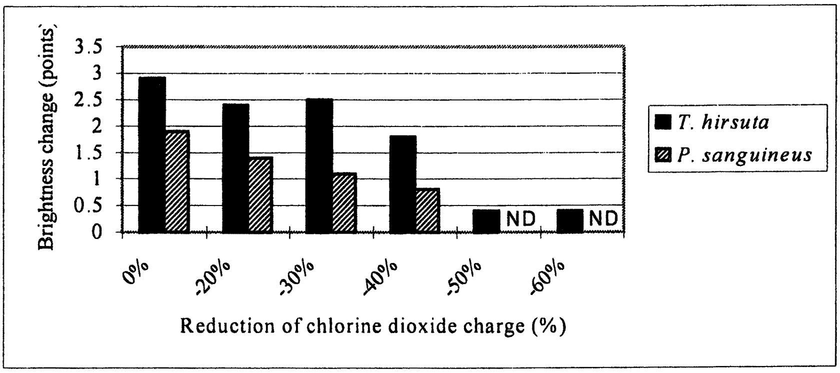

Figure 12: Impact of LE- pretreatment on reduction of ClO_2 charges used in D_1ED_2 bleaching of post-oxygen soda pulp

Effect of L-treatment on Viscosity of Pulps

Following LE-treatment, the viscosity of post-oxygen kraft pulp was 42.25 mPa·s (with *T. hirsuta* laccase) and 37.00 mPa·s (with *P. sanguineus* laccase) (Table II). Following LE-DED bleaching with full chlorine dioxide charges, the viscosity values generally decreased. However, in the case of *T. hirsuta* laccase, there was a viscosity increase of 3.25 mPa·s over the DED-bleached control (36.75 mPa·s). At the same time the viscosity of post-oxygen kraft pulp, bleached with the *P. sanguineus* laccase, decreased by 5.75 mPa·s over the same control.

A similar pattern was observed when comparing viscosities of post-oxygen soda pulp and unbleached sulfite pulp bleached with the two laccases (Table II). The slight increase in the viscosity of the *T. hirsuta* laccase-bleached pulps is

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indicative of the selective lignin oxidation occurring during the laccase/HBT treatment which leaves the cellulosic component of the pulp intact. Similar findings were reported using the same enzyme/HBT system on pine kraft pulp (17). In contrast, the reduced viscosity of pulps pretreated with *P. sanguineus* laccase/HBT pointed to cellulose degradation. This was apparently due to a non-selective action of the *P. sanguineus* laccase/HBT system since no cellulase activities were detected in the crude enzyme.

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Table II. Impact of L-treatment with *T. hirsuta* laccase/*P. sanguineus* laccase on viscosity (mPa·s) of pulps

	Pulps		
Treatments	*Kraft*	*Sulfite*	*Soda*
None	47.20	33.75	18.30
-L+E	45.50	25.25	17.00
+L+E	42.25[a]/37.00[b]	25.00[a]/21.25[b]	15.25[a]/13.00[b]
-L+E+DED	36.75	21.75	14.00
+L+E+DED	40.00[a]/31.00[b]	22.00[a]/19.00[b]	14.00[a]/12.00[b]
DED	36.75	22.00	13.00

NOTE: L: Laccase/HBT treatment; E: Alkaline extraction; D: Chlorine dioxide; For details, see Materials and Methods; [a]*T. hirsuta* laccase/HBT treatment; [b]*P. sanguineus* laccase/HBT treatment.

Conclusions

Both the microbial origin of the laccases and the pulp source influenced the delignification and bleaching performance of the LMS. There was, however, no obvious correlation between the stability of the enzyme against inactivation due to HBT and its effectiveness in the L-treatments. For instance, the laccase from *P. sanguineus* was shown to be the most stable enzyme, however, its efficiency in pulp delignification and bleaching was lower than the less stable *Trametes* laccases.

Best results on all pulps in terms of kappa number reduction were obtained with the two *Trametes* laccases, followed by the *P. sanguineus* laccase and finally the laccase from *Peniophora.* Pulp delignification decreased in the following order (the best kappa number reductions are shown in brackets): unbleached hardwood sulfite pulp (51%)>post-oxygen softwood kraft pulp (29%)>post-oxygen hardwood soda pulp (22.7%)>unbleached softwood kraft pulp (21.3%). The significant differences between these results suggest that factors such as wood species, method of pulping and pulp pretreatment could impact the pulp susceptibility to the LMS and the effectiveness of the LMS treatments.

With respect to brightness gains following LE-treatment, the pulps responded in the following descending order: post-oxygen softwood soda pulp (9.4 points)>unbleached hardwood sulfite pulp (3.2 points)>post-oxygen softwood kraft pulp (2.3 points)>unbleached softwood kraft pulp (1.1 points). The brightness loss which occurred in some cases could be due to the formation of chromophoric structures during the oxidation of lignin. Apparently these chromophores are resistant to a mild alkaline extraction and need harsher conditions to be removed.

Significant brightness improvements were obtained in the LE-DED bleaching of post-oxygen softwood kraft pulp (12.2 points). Savings of chlorine dioxide of 40% (post-oxygen softwood kraft pulp), 50% (unbleached hardwood sulfite pulp) and 60% (post-oxygen softwood soda pulp) appeared to be possible without affecting negatively the final pulp brightness. This in fact clearly indicates the tremendous bleaching potential of the LMS as compared to xylanases where the average reduction of the total chlorine consumption during bleaching generally does not exceed 15% (22).

Biobleaching with LMS can affect pulp viscosity depending on the selectivity of action of the particular laccase/HBT system used. Overall, the delignification abilities of the LMS, as expressed in the LE experiments, have been verified in the subsequent ECF bleaching trials. Therefore, a simple LE-treatment of pulp could be used as an initial indicator in the screening for more efficient LMS.

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Chapter 28

Formation of Quinonoid Structures in Laccase-Mediator Reactions

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A softwood kraft pulp (kappa # 71.4) was subjected to a series of laccase-mediator treatments using 1-hydroxybenzotriazole (HBT), *N*-acetyl-*N*-phenylhydroxylamine (NHA), and violuric acid (VA). Based on the experimental conditions used in this study, the highest delignification response was observed with VA. Losses in brightness were observed after all three LMS systems, and were attributed to the formation of quinonoid structures. The residual lignins were isolated and derivatized with trimethylphosphite. The ^{31}P NMR spectral analyses confirmed the formation of quinones from $LMS_{VA,NHA,HBT}$. The decrease in quinones after an LMS(E) could be attributed, in part, to a Michael addition of OH^- to quinones.

This paper is dedicated to
Selim R. Chakar and Iziderius D. Ragauskas

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Introduction

Over the last two decades, research efforts in pulping and bleaching have largely focused on environmental issues. As these concerns continue to be addressed, new research opportunities are developing. One area of active research focuses on improving pulp yields from pulping and bleaching operations [1-7]. The benefits in enhancing pulp yields are fourfold, including improved wood utilization practices, reduced pulp manufacturing capital costs, reduced operating costs, and improved profitability. An attractive approach for improving pulp yields consists of halting the kraft cook prior to reaching the terminal phase. In the terminal phase of pulping, the selectivity between lignin removal and carbohydrate degradation is significantly reduced resulting in loss of pulp carbohydrates. Stopping a kraft cook prior to the terminal phase reduces carbohydrate losses but yields a pulp with high lignin content (pulp kappa number of 40-50). A promising strategy for removing the lignin from such pulps prior to bleaching consists of employing a single- or a double-oxygen stage. Several research groups have reported that pulp yields can be increased in the range of 2-6% by employing this approach [2,5,6].

Recently, we have begun investigating the potential of employing lignin degrading enzymes, more specifically, laccase-mediator systems (LMS), to delignify high kappa kraft pulps [8,9]. Laccase has been shown to effectively oxidize phenolic compounds and phenolic lignin model compounds [10-15]. However, laccase alone is ineffective in delignifying pulp fibers [16]. This inefficacy is attributed to the size of the enzyme and, hence, to its inability to diffuse in a pulp fiber and oxidize the lignin [17]. The limitations of diffusion were shown to be circumvented by the addition of low molecular weight compounds, also known as mediators. Bourbonnais et al. first demonstrated this approach when they reported that laccase in the presence of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) could delignify kraft pulps [18]. In addition, based on model compound studies, it was shown that the specificity of the laccase-mediator system could be expanded to non-phenolic substrates [19-21]. The proposed mechanism for this mediator assisted biodelignification process involves laccase oxidizing ABTS and, in turn, the oxidized ABTS diffuses into the pulp fiber and reacts with the lignin. The reduced ABTS is then reoxidized by laccase. Since then, a host of alternative mediators have been reported in the literature [22-24]. Some of the more effective mediators are N-hydroxybenzotriazole (HBT), violuric acid (VA), and *N*-acetyl-*N*-phenylhydroxylamine (NHA). Typically, these mediators have been employed with laccase on low-lignin content kraft and sulfite pulps, as well as on lignin model compounds [24-36].

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The ability of these LMS treatments to remove lignin from high-lignin content pulps has remained largely unexplored. We have recently demonstrated that an LMS treatment, using HBT or NHA as mediators, can remove significant amounts of lignin from high-kappa kraft pulps [8,9]. NMR analysis of the residual lignin in the pulp after an LMS treatment indicated that the bio-delignification treatment extensively oxidizes C-5 noncondensed phenolic lignin structures, whereas C-5 condensed phenolic lignin structures were overall resistant to oxidation. In addition, side chain oxidation did occur on the propane-linking unit of lignin. The primary product detected from these oxidative treatments has been the formation of carboxylic acid groups [8,9].

The presence of quinone groups in an LMS treated pulp has been frequently proposed [8-9,28,30,36-37]. Lignin model compounds studies with laccase indicate that this can occur [38]. Recently, Poppius-Levlin et al. [28,39] presented FT-IR data suggesting that the residual lignin from LMS treated pulp has an enriched level of quinonoid structures.

The formation of quinones in LMS treated pulps could readily explain the substantial increases in brightness when a kraft pulp is first subjected to LMS and then treated with alkaline hydrogen peroxide [8-9,36-37]. It is well established that alkaline hydrogen peroxide readily reacts with para and ortho-quinones [40,41]. The removal of these intensively colored bodies from kraft pulp with alkaline peroxide would significantly improve the brightness of the pulp. The purpose of this study was to determine the relative amounts of quinones in residual lignins isolated from a softwood high-kappa kraft pulp before and after LMS treatments, using HBT, NHA, and VA as mediators.

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Experimental

Methods and Materials

Chemicals. All chemicals were purchased from Aldrich Co., Milwaukee, WI, and used as received, except for *p*-dioxane, NHA, and laccase. *p*-Dioxane was freshly distilled over $NaBH_4$ prior to using it for lignin isolation experiments. NHA was synthesized in accordance with Oxley's method [42]. Laccase from *Trametes villosa* was donated by Novo Nordisk Biochem, Franklinton, NC.

Furnish. The softwood kraft pulp employed in this study originated from a 25-year-old *Pinus taeda* tree that was donated by Union Camp (now International Paper), Savannah, GA. The chips were cooked at Potlatch Corp. facilities in Cloquet, MN, to an H-factor of 573 using 18.5% active alkali. The

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pulp was thoroughly washed, screened, centrifuged, fluffed, and stored at 4°C. Prior to executing the experimental design called for in this study, the pulp was Soxhlet extracted with acetone for 24 hours and then thoroughly washed with distilled water to remove the residual acetone.

Hexenuronic acid in pre-acetone extracted brownstock. The content of hexenuronic acids in the brownstock was indirectly measured in accordance with a modified procedure reported by Vuorinen et al. [43]. In brief, a 1000-mL round bottom flask was charged with 25 g of pulp (o.d. basis). The pulp consistency was adjusted to 3% by adding distilled water. The pH was then lowered to 3 using 4.0 N sulfuric acid. The slurry was refluxed for three hours at 100°C. The change in kappa number before and after the treatment was then determined and served as an indirect measurement of hexenuronic acids (see **Table I**).

Table I. Changes in Kappa # After Acid Treatment of Brownstock

Replicate #	Initial Kappa	Final Kappa	% Change
1	73.4	71.5	2.6
2	73.4	71.9	2.0

Laccase assay. Laccase activity was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity (U) was defined as the change in absorbance at 530 nm of 0.001 per minute per milliliter of enzyme solution, in a 100 mM potassium phosphate buffer (2.2 mL) and 0.216 mM syringaldazine in methanol (0.3 mL, pH 6.7). The procedure was carried out at 23°C. The activity of the laccase was 1.87E + 06 U/mL of enzyme solution.

Laccase-mediator delignification procedure (LMS). A 2000-mL capacity Parr reactor equipped with a stirrer, a pressure gauge, a heating mantle, and connected to a Parr 4842 temperature controller was charged with 60 g of o.d. fibers. The pulp consistency was adjusted to 5% with distilled water. The slurry was then heated to 45°C and was maintained at this temperature throughout the incubation period. VA (4.4 mmol/10g of o.d. pulp, or NHA, or HBT) was then added to the heated slurry. Subsequent to mixing the slurry (approx. 5 minutes), the pH was adjusted to 4.5 with glacial acetic acid or saturated sodium bicarbonate solution. Laccase (93,500 U, or 0.05 mL of enzyme solution/g of o.d. fiber) was added, and the reactor was sealed and pressurized with oxygen to 145 psig. After a mixing period of 1 hour, the pulp was removed from the reactor and thoroughly washed with distilled water (12 L per 10 g of o.d. pulp). The treated and washed pulp was either followed by a subsequent alkaline extraction stage or simply used as is.

Alkaline extraction stage (E). Alkaline extractions were performed in 4 mm-thick Kapak heat sealable pouches for 1 hour, at 80°C, and 10% consistency. All E treatments employed 2.5% charge of NaOH.

Control experiments in the absence of laccase (MS). Control experiments were performed in the absence of laccase and in the presence of VA, HBT, and NHA. These treatments were carried out in accordance with the laccase-mediator delignification procedure described above, except no laccase was employed.

Pulp characterization. The brownstock, MS, LMS, and LMS(E) pulps were characterized for kappa number and brightness in accordance with standard TAPPI Standard Methods T236-cm85 and T452-om98, respectively.

Isolation of residual lignins. The isolation of residual lignins was carried out following standard literature methods [44-46]. In summary, a 5000-mL three-necked round bottom flask was charged with 50 g of o.d. pulp and the consistency was adjusted to 4% by adding a 0.10 N HCl 9:1 *p*-dioxane:water solution. The slurry was then refluxed for two hours under an argon atmosphere. The pulp was filtered and the filtrate was filtered through celite, neutralized, and concentrated under reduced pressure to approximately 10% of the original volume. Water (approx. 400 mL) was added and the mixture was concentrated again under reduced pressure to remove the last traces of *p*-dioxane. The solution's pH was then adjusted to 2.5 with 1.00 N HCl. The precipitated lignin was collected, washed several times, and freeze-dried. Lignin yields ranged from 46.3 to 49.0%. Lignin yields were calculated as follows: % lignin yield = {mass of lignin isolated/ (initial kappa of brownstock x 0.15)}x100. The calculated lignin yields were corrected for initial hexenuronic acid groups content.

Derivatization of residual lignins with trimethylphosphite (TMP). Derivatization of residual lignins with trimethylphosphite was performed in accordance with Zawadzki's method [47,48]. In brief, a 30 mg sample of lignin previously dried at 40°C under vacuum for 24 hours was treated with 500 μL of 50% TMP/DMF (v/v) under an argon atmosphere for 7 days. Subsequent to the incubation period, excess trimethylphosphite was removed by first adding 250 μL of DMSO and then placing the lignin solution under vacuum at 45°C until the sample was nearly dry (approx. 6 hours). The treated

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lignin samples were then dissolved in 500 μL 60% of DMSO-d_6/pyridine (v/v) containing tri-meta-tolylphosphate (0.7 mg/mL) and chromium–acetylacetonate (0.9 mg/mL). Deionized water (5 μL) was then added and the lignin solution was allowed to mix for 12 hours prior to acquiring the ^{31}P NMR spectrum.

^{31}P NMR of derivatized residual lignins. ^{31}P NMR spectra of derivatized lignins were acquired using a 90° pulse, a 5-second pulse delay, inverse-gated broad-band proton decoupling and 1000 scans per spectrum (approx. 1 hr 36 min total acquisition time) [47,48]. All ^{31}P NMR spectra were recorded on a DMX 400 MHz Bruker spectrometer.

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Results and Discussion

Extent of Delignification and Brightness

The delignification and brightness responses of laccase-mediator systems employing HBT, NHA, and VA on a softwood kraft pulp (kappa # 71.4) were evaluated before and after an alkaline extraction stage (E). In addition, a series of control experiments in the absence of the laccase were carried out. The kappa and brightness measurements relative to the initial brownstock are summarized in **Table II**.

Table II. Kappa and TAPPI Brightness for a softwood kraft pulp treated with MS[a], LMS[b] and LMS(E)[c] using HBT, NHA, and VA as mediators[d].

Pulp/Treatment	Kappa #	St.dev	TAPPI Brightness	St.dev
Brownstock	71.4	0.19	18.4	0.11
MS_{NHA}	71.3	0.11	18.5	0.20
MS_{HBT}	71.0	0.18	18.7	0.33
MS_{VA}	71.2	0.08	18.5	0.15
LMS_{NHA}	67.0	0.29	7.8	0.43
LMS_{HBT}	65.3	0.11	11.6	0.45
LMS_{VA}	53.6	0.09	9.8	0.39
LMS_{NHA}(E)	58.4	0.37	10.7	0.21
LMS_{HBT}(E)	57.4	0.01	15.5	0.35
LMS_{VA}(E)	45.1	-	13.7	0.37

[a] MS treatment in the absence of laccase but in the presence of mediator.
[b] LMS treatment in the presence of both laccase and mediator.
[c] LMS(E) treatment in the presence of both laccase and mediator and followed by an alkaline extraction stage (E).
[d] see experimental section for details.

The results clearly indicate that in the absence of laccase and in the presence of the mediator only, delignification did not occur. In addition, previous LMS studies have demonstrated that the delignification response of a laccase treatment in the absence of a mediator is insignificant [16,26]. Hence, both the mediator and the laccase must be present in order to achieve delignification. Based on the experimental conditions employed in this study, VA was a superior mediator with respect to HBT and NHA on this high-kappa kraft pulp. The extent of delignification of both NHA and HBT was comparable.

It is well known that a high content of hexenuronic acids (HexA) has an adverse impact on the kappa number since HexA consume potassium permanganate [43]. As summarized in **Table I**, the change in kappa number after the acid stage was approx. 2%, implying that the HexA content is insignificant and that the kappa numbers in this study were a good reflection of the lignin content.

Accompanying the LMS delignification, the treated pulps suffered a loss in brightness. The brightness data shown in **Table II** indicate that the LMS treatment always darkens the pulp with respect to the brownstock. This effect was most significant with NHA and VA. The extraction stage with sodium hydroxide improved the final brightness of the LMS treated pulps relative to the brownstock; however, it never exceeded the initial brightness. Based on our previous studies [9,36], we have shown that this darkening effect can be further alleviated with the reinforcement of the extraction stage with peroxide, and with peroxide and oxygen. This effect is consistent with the proposed quinone chemistry of an LMS stage.

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Quinone Content. The role of quinones in the observed LMS delignification chemistry was explored by isolating the residual lignin from the SW kraft brownstock, and after the MS, LMS, and LMS(E) treatments, as described in the experimental section. The combined content of ortho- and para-quinones was examined using a trimethylphosphite derivatization procedure and ^{31}P NMR. Studies by Zawadzki and Ragauskas [48-51], Argyropoulos and Zhang [52], and Zhang and Gellerstdedt [53] have shown that trimethylphosphite can readily be used to tag ortho- and para-quinones and after hydrolysis yield a stable phosphate ester adduct. This adduct is detected via ^{31}P NMR experiments and is a means to establish a semi-quantitative relationship of the quinone content. The combined ortho- and para-quinone data are presented in **Figure 1**.

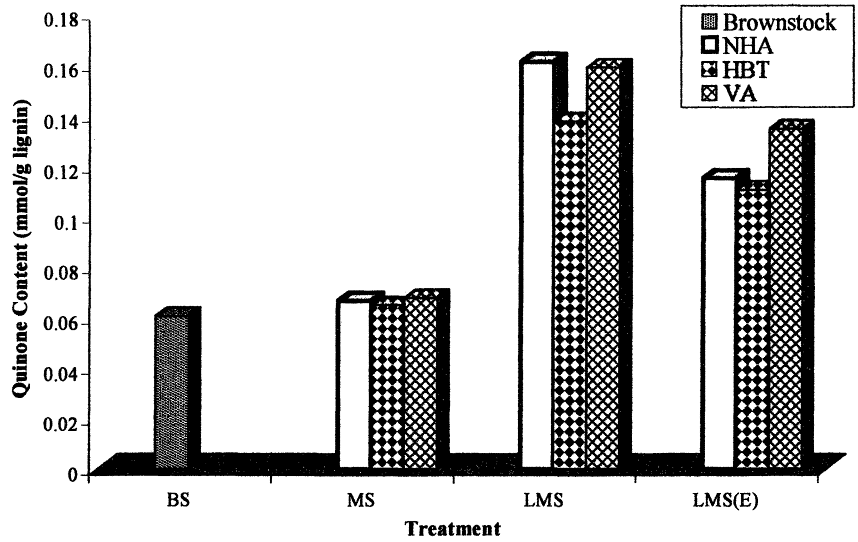

Figure 1. Semiquantitative quinone content of residual lignins isolated from the brownstock (BS) and after MS, LMS, and LMS(E) treatments using NHA, HBT, and VA as mediators.

The experimental results indicate that the content of quinone structures in the brownstock is minute. This value is comparable to that reported by Zawadzki [50]. Treatment of the pulps in the presence of mediators and oxygen failed to introduce any further quinones into the residual lignins.

Repeating these experiments in the presence of both laccase and mediator led to an approximate 2.7-fold increase in detectable quinone structures when NHA and VA were used. The relative trend also suggests that the content of quinones was lower when HBT was employed.

The subsequent alkaline extraction stage reduced the quinone content, on average, by approximately 21%. The loss of quinones during the alkaline extraction stage can be attributed to the reactivity of NaOH with such structures. The nucleophilic addition of OH^- to quinonoid structures can result in increased solubility. This type of chemistry can lead to the formation of hydroxy substituted catechols *via* a Michael addition of hydroxide anions and also to alpha-hydroxy-carboxylic acid cyclopentadiene structures (see Figure 2). The latter structures are postulated to stem as a result of a nucleophilic addition of OH^- followed by a benzylic acid rearrangement [54].

Despite the loss in quinone structures subsequent to the extraction stage, the data suggest that the residual lignin still contained approximately 50% more quinone structures than the brownstock. One possible explanation for

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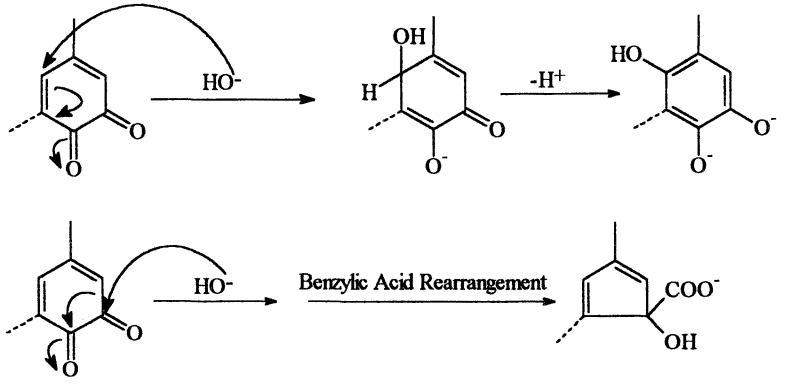

Figure 2. Proposed sites of addition of hydroxide anions to quinone structures [54,55].

this observation could be attributed to the proposed propensity of *o*- and *p*-benzoquinones to undergo condensation reactions leading to the formation of bi-phenyl linkages [55]. As a result, the solubility of such structures may be adversely affected. Another possible explanation may be linked to the ability of catechols to readily be oxidized back to quinone structures.

Conclusions

In summary, this study provides some of the first spectroscopic data that establishes conclusively the formation of quinones in LMS and LMS(E) treated softwood kraft pulps. The data provide an explanation, in part, for the darkening of kraft pulps after an LMS stage and its subsequent partial brightening after an LMS(E) stage. The observed formation of quinones after an LMS stage is also consistent with the reported brightness benefits of alkaline peroxide bleaching of LMS treated pulps. The formation of quinones and the darkening effect of pulps are important aspects of the chemistry of LMS delignification. This issue will need to be addressed and further understood if LMS technology is to be implemented commercially.

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Chapter 29

Cellobiose Dehydrogenase as a Ligninase

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Cellobiose dehydrogenase is an extracellular fungal enzyme that can indirectly generate hydroxyl radicals by reduction of Fe^{3+} to Fe^{2+} and O_2 to H_2O_2 under oxidation of cellobiose. We have studied the effects of such a CDH system on lignin model compounds using GC-MS as the analytical method. The results indicate the following effects on lignin: 1) Lignin can be depolymerized by cleavage of ß-aryl-ether-linkages. 2) Non-phenolic lignin structures can be converted to phenolic lignin groups by demethylation and possibly hydroxylation. Phenolic lignin structures can be oxidized by the manganese peroxidase (MnP) system. We demonstrate that the MnP system can oxidize a non-phenolic compound that had been modified by the CDH system, suggesting that CDH and MnP form an extracellular pathway in lignin biodegradation. We also demonstrated that CDH can depolymerize synthetic lignin to some extent.

In Oxidative Delignification Chemistry; Argyropoulos, D.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Lignin is a polymer of phenyl propanoid units and is unusual among biomolecules due to its complex and racemic structure. The monomers are connected with several different types of ether and carbon - carbon linkages. The aromatic ß-O-4 ether is the most common (*1*). In woody tissues the lignin is deposited in the middle lamellae, where it is the dominant compound, and also in the primary and secondary cell walls. The lignin increases the hydrophobicity, incompressibility and rigidity of wood and makes a lignified cell wall impermeable to enzymes (*2*). Thus, the lignin acts as an obstacle to microbial degradation of woody tissues, and efficient lignin biodegradation is limited to some filamentous fungi and some groups of bacteria (*3-5*). The ligninolytic system of Basidiomycetic white-rot fungi is the best known group of efficiently ligninolytic organisms, although brown-rot and soft-rot fungi can degrade lignin to some extent (*3-5*). Since extracellular enzymes can not come into direct contact with the lignin (with the exception of lignin on surfaces), it is believed that at least the initial lignin degradation is carried out by low molecular weight compounds, *redox mediators*, that are activated by extracellular redox enzymes and possibly also by the fungal mycelium (*4,6,7*). Mn(III), various aromatic radicals and OH•, O_2^{-}• and other activated oxygen species have been suggested to work as redox mediators (*3-5,7*). Mn(III) in particular can be generated by the hydrogen peroxide-dependent oxidation of Mn(II) (complexed with, for instance, oxalic acid) by the extracellular heme-enzyme manganese peroxidase (MnP, EC 1.11.1.7) (*8*) (Figure 1). This enzyme is widespread among white-rot fungi and it has been suggested to be a key enzyme in lignin biodegradation. However, native lignin consists of a mixture of phenolic and non-phenolic structures, where the latter are the most abundant (1), and Mn(III) can not in general oxidize the non-phenolic lignin structures directly (*9*). Other enzymes or cofactors are thus probably involved. The presence of unsaturated fatty acids seems to allow the MnP system to also oxidize also non-phenolic structures (*10*). Another extracellular peroxidase from white-rot fungi, lignin peroxidase (LP, EC 1.11.1.7), can directly oxidize non-phenolic lignin model compounds and a cofactor to the enzyme produced by the fungus, veratryl alcohol, has been suggested to work as a redox mediator in the form of a phenoxyradical (*11,12*). However, the lifetime of this radical is so short that it is

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questionable whether it can work efficiently as a redox mediator (*13*). Laccase is a copper-containing oxidase that is secreted by many fungi. In the white-rot fungi *Pycnoporus cinnabarinus* it has been suggested to use the fungal metabolite 3-hydroxyanthranilate as redox mediator in lignin degradation (*7*) and in the white-rot fungus *Trametes versicolor* other substances have been suggested (*14*). Generally, laccases are too weak oxidants to directly oxidize non-phenolic lignin structures (*7,15*).

Cellobiose dehydrogenase (CDH) is another enzyme produced by many wood-degrading fungi. The enzyme was earlier called cellobiose oxidase (EC 1.1.3.25). This extracellular flavocytochrome is a ping-pong type redox enzyme that oxidizes cellobiose, a product of enzymatic cellulose degradation, to cellobionolactone and can reduce a large number of electron acceptors, including complexed Fe^{3+}, O_2 (to H_2O_2, the reaction is slow), quinones and phenoxy radicals (*16-20*). The enzyme has been suggested to be involved in lignin biodegradation by reducing the reaction products of ligninolytic peroxidases (*21*). It is however unclear how these reactions should promote lignin degradation, since CDH just catalyzes the opposite reaction to lignin peroxidase and laccase. Inhibition of polymerization of phenoxy-radicals can be one answer (*21*). Another suggestion is that the function of CDH is to generate Fe^{2+} and possibly H_2O_2 that later can react in a Fenton type reaction forming hydroxyl radicals (*18*). The hydrogen peroxide may also be produced by for instance glyoxal oxidase (*22*). Since both H_2O_2 and Fe^{2+}, chelated with for instance acetic acid, are small and can easily diffuse, OH• can be formed far away from the CDH inside narrow pores that enzymes can not penetrate. In this work we summarize our studies of the possible lignin depolymerizing and modifying effects of CDH generated hydroxyl radicals. We have shown that CDH, in the presence of ferri acetate, cellobiose and hydrogen peroxide, can depolymerize synthetic lignin to some extent (*23*). Furthermore, recent studies with CDH and non-phenolic lignin model compounds suggest that CDH may cleave ß-O-4 ethers and perform demethoxylation in non-phenolic lignin under simultaneous introduction of hydroxyl groups (*24*). This modification "activates" the lignin allowing the modified structure to be oxidized by Mn(III) generated by MnP (*25*). These data suggest a

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role for CDH in conjugation with the MnP system in initial lignin biodegradation.

Material and Methods

Enzymes

CDH was purified from a cellulolytic culture filtrate of the white-rot fungus *Phanerochaete chrysosporium* strain K3 (*26,27*) as described in (*28*). MnP from *P. chrysosporium* was obtained from Tienzyme Inc. (USA). Both proteins were homogeneous in SDS PAGE analysis.

Depolymerization of Synthetic Lignin (*23*)

Radioactive (ring - U-^{14}C) synthetic lignin with a molecular weight of 4 000 - 10 000 Da was prepared as described earlier (*5,23*). The specific activity was 277 kBq/mg. Samples of 833 Bq were incubated in 3 ml with 92 nM CDH, 0.2 mM ferri acetate 0.5 mM cellobiose and 0.5 mM hydrogen peroxide, for 16h at 30°C in 50 mM ammonium acetate buffer, pH 4. Various control mixtures where one or several of the ingredients had been omitted were incubated in parallel. The samples were filtered through new ultrafiltration membranes with a cut-off of 500 Da. The radioactivities of the filtrates were measured in a Tri-Carb liquid-scintillation analyzer model 1600 CA from Packard.

Treatment of Model Compounds

0.39 µM CDH was incubated in 50 mM ammonium acetate buffer, pH 4.0 at 35°C with 8 mM of a non-phenolic lignin model compound (3,4-dimethoxyphenyl glycol (veratrylglycol)) and 5 µM ferri acetate for 24 h in a final volume of 1 ml. Amounts of 2 µmole cellobiose and 1 µmole hydrogen peroxide were added every 8th hour. The samples were filtered on a Nanosepp 10 kD cut off membrane and the supernatants were freeze dried. Samples were

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analyzed with gas chromatography coupled to mass spectroscopy (after silylation) or HPLC, or subjected to treatment with MnP. Various control mixtures where one or several of the ingredients had been omitted were incubated in parallel and one with 1 mM desferrioxamine mesylate (that prevents reduction of Fe^{3+} by CDH (*17*)) included in the compete mixture.

The treatment with MnP was carried out as follows: Mixtures containing 8 mM model compound 0.2 u/ml MnP and 0.1 mM Mn(II)oxalate, were incubated in 10 mM ammonium acetate buffer, pH 5 at 35°C. Hydrogen peroxide was added in 30 portions of 0.1 µmole separated by 15 minutes (to protect MnP from inactivation). Samples of a non-phenolic model compound (veratrylglycol), with or without pretreatment with the CDH system, and a phenolic lignin model compound (α-methylsyringyl alcohol), were subjected to this treatment. The material was filtered through a Nanosepp membrane and freeze dried as described above, and analyzed with HPLC, GC/MS and spectroscopic analysis as described bellow.

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Spectroscopic Analysis

The active species in the MnP system, Mn(III)oxalate, has an absorbance maximum close to 500 nm (*29*). If MnP, Mn(II)oxalate and hydrogen peroxide are mixed in a cuvette an increase in absorbance at 500 nm is obtained. After some seconds the absorbance stabilizes at an approximately constant level due to disproportionation of Mn(III) to Mn(II) and Mn(IV). If a molecule that can be oxidized by Mn(III) is added to the system, the increase of absorbance at 500 nm is inhibited. Thus, it is possible to determine if a compound is oxidisable for the MnP-system with a simple spectrometric test (*30*). The test was carried out as follows: MnP (0.15 u/ml), Mn(II)oxalate (1.6 mM), the substance that should be tested (to 1 mM) and hydrogen peroxide (0.1 mM - added as the last component). The final volume was 500 µl. Absorbance at 500 nm is recorded in a time scan mode. The non-phenolic model compound was tested with and without pretreatment with the CDH system. As a positive control, the phenolic lignin model compound α-methylsyringyl alcohol was also tested.

GC/MS

Samples for mass spectroscopy (MS) were silylated as follows: Dried material was dissolved in anhydrous pyridine at a concentration of 8 mg/ml. Eight mg of Silylation kit (N,O-bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane (BSTFA + TMCS), 99:1) were added and the sample was incubated at 70°C for 1 h. The sample was then dried under nitrogen and dissolved in dried dichloromethane.

The analysis was carried out on a Hewlett Packard 5890A gas chromatograph (GC), linked to a VG 70-250SE high resolution spectrophotometer. High purity helium was used as carrier gas at a flow of 2 ml/min. For the GC separations a fused silica capillary column (CP SIL 8CB/MS, 60 m) was used. The column had an inner diameter of 0.32 mm and a film thickness of 0.25 mm. In all GC-analyses the temperature was 50°C for 2 minutes. Thereafter it was increased by 5°C per minute until the temperature reached 310°C. This temperature was held for 6 minutes. The MS interface temperature was 290°C. A sample volume of 1 ml was injected splitless. The MS instrument was operated in the electron ionization (EI) mode using an electron energy of 70 eV. The resolution was set to 1000 and the ion source temperature was held at 180°C. A scan range of 40 - 700 m/z and a scan rate of 1 scans/s were used in the study.

RP HPLC

Reverse phase (RP) HPLC separations were carried out on a HICHROM 5C-18 column (4.6 x 140 mm) at a flow rate of 1 ml/min. Samples of untreated or enzyme treated model compounds were dissolved in a water-methanol mixture (50/50) and a volume of 30 ml was injected into the column. The system was washed with water containing 0.1% trifluoroacetic acid for 0.5 minutes and then with an increasing linear gradient of methanol with 0.1% trifluoroacetic acid (0 to 100%) for 5 minutes and eventually with methanol with 0.1% trifluoroacetic acid. Absorbance at 280 nm was monitored.

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Results and Discussion

To investigate whether CDH could depolymerize lignin, samples of synthetic radioactive lignin were incubated with CDH in the presence of hydrogen peroxide, ferri acetate and cellobiose. The mixture was thereafter filtered through a membrane with a molecular weight cut-off of 500 and the amount of radioactivity that had passed through the membrane was measured. Controls, in which specific components of the reaction mixture had been omitted, were incubated in parallel with the sample. The result is presented in Table I (*23*).

Table I. Degradation of Synthetic Lignin

Incubation mixture	Fraction of synthetic lignin passing through a 500 cut off membrane (percentage)
Only acetate buffer	3.60
hydrogen peroxide	3.77
hydrogen peroxide, ferri acetate	4.35
CDH, cellobiose, ferri acetate	5.40
CDH, cellobiose, ferri acetate, hydrogen peroxide	6.60

Experiment carried out as described in Materials and Methods. Results are from (*23*). This result is a typical result from several repeats with membranes of different cut off.

Samples that had been incubated with the intact CDH system (CDH, cellobiose, ferri acetate and hydrogen peroxide) could pass through the membrane to a significantly higher extent than the controls (Table I). Note the fact that lignin depolymerization seemed to occur even without added hydrogen peroxide. This is probably as a result of hydrogen peroxide formation by CDH itself during the oxidation of cellobiose (*16,17*). It appears that CDH can depolymerize synthetic lignin by generation of hydroxyl radicals, although formation of other radicals can not be totally excluded. The sample that had been incubated with only ferri acetate and hydrogen peroxide might also have been somewhat depolymerized.

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This can be due to a slow spontaneous formation of OH• (or other radicals) by this system.

Although this depolymerization of lignin by CDH is most likely dependent on hydroxyl radicals, the exact mechanism of the reaction is not known and neither which lignin structures that are attacked. Therefore, we performed an experiment with a simple model compound for non-phenolic lignin, veratrylglycol (3,4 dimethoxyphenyl glycol) (Figure 1) (*23*). The model compound was incubated with CDH in the presence of cellobiose, ferri acetate and hydrogen peroxide as above. After incubation the mixtures were filtered through a 10 kDa cut off membrane to remove CDH and analyzed with GC/MS. This analysis indicated that a product had been created where the model compound had been demethylated (or rather been demethoxylated and hydroxylated) by the CDH system (Figure 2). A small amount of a didemethylated product was also detected. Control experiments in which specific components of the reaction mixture had been omitted showed that the same reaction products, although in smaller amounts, were formed by a mixture where hydrogen peroxide had been omitted. This is probably because CDH itself can form hydrogen peroxide. Traces were also detected in the control without CDH. The latter can be explained by spontaneous formation of OH• in an H_2O_2 system as discussed above.

In Figure 1 a possible mechanism for the demethylation of veratryl glycol by CDH formed hydroxyl radicals, is shown. If the CDH system reacts with lignin in a similar way, the biopolymer will be attacked at aromatic ether linkages by the CDH system. This reaction can be responsible for the lignin depolymerization in the above experiment (Table I). Note that this system can attack non-phenolic lignin structures in contrast to the MnP system, that alone can only attack phenolic lignin structures. According to the mechanism suggested in Figure 1, both ether links between lignin monomer residues and demethoxylations can be the effect of the reaction between the aromatic rings in lignin and CDH-generated OH•, depending on where the hydroxyl radical reacts. In both cases a hydroxyl group is introduced, i.e., non-phenolic lignin is converted to phenolic lignin, that can be oxidized by Mn(III) generated by MnP. To test this possibility we treated the non-phenolic model compound with the CDH system followed by the MnP system (*25*). The results were analyzed with GC/MS and HPLC.

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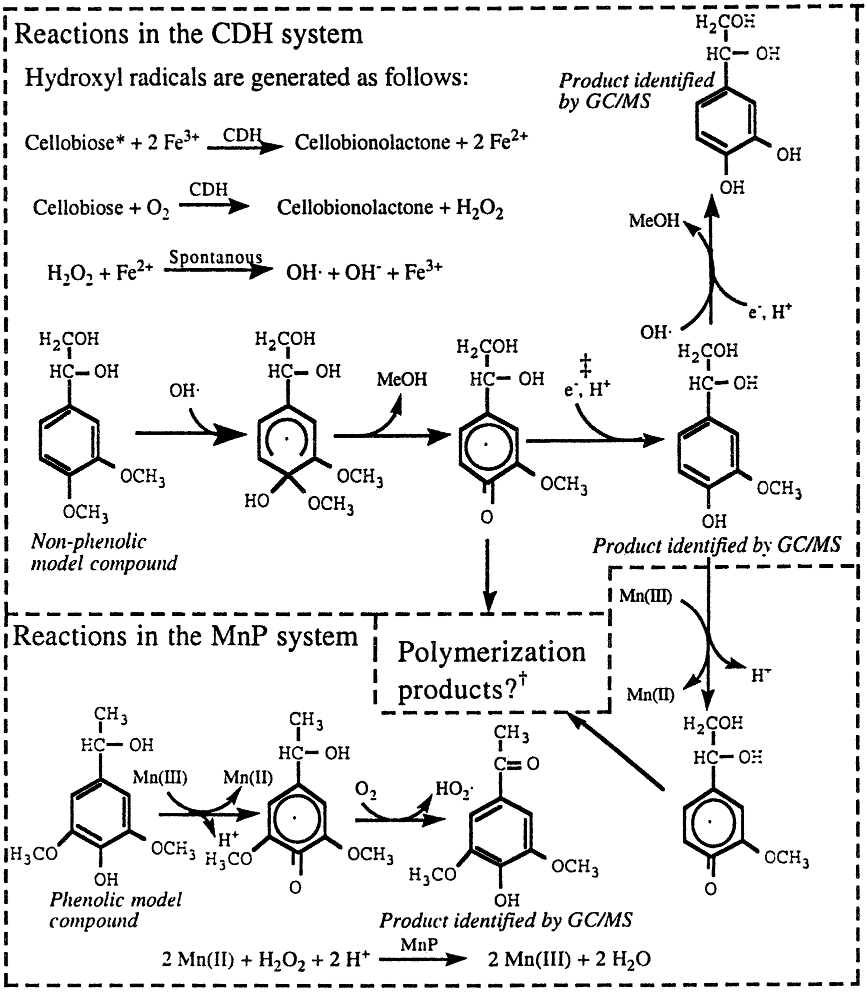
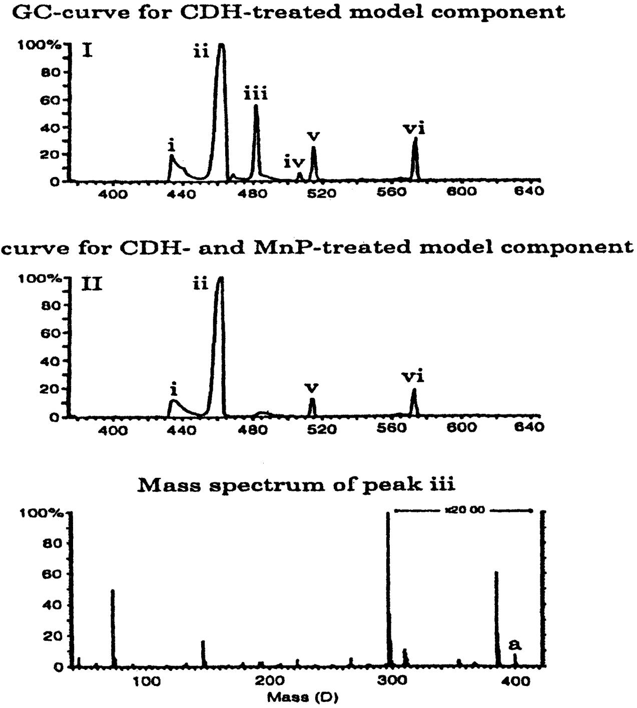

Reactions in the CDH system
Hydroxyl radicals are generated as follows:
Cellobiose* + 2 Fe^{3+} $\xrightarrow{CDH}$ Cellobionolactone + 2 Fe^{2+}
Cellobiose + O$_2$ $\xrightarrow{CDH}$ Cellobionolactone + H$_2$O$_2$
H$_2$O$_2$ + Fe^{2+} $\xrightarrow{Spontanous}$ OH· + OH$^-$ + Fe^{3+}
Non-phenolic model compound
OH·
MeOH
‡
e$^-$, H$^+$
Product identified by GC/MS
Product identified by GC/MS
Reactions in the MnP system
Polymerization products?†
Mn(III)
Mn(II)
H$^+$
O$_2$
HO$_2$·
Phenolic model compound
Product identified by GC/MS
2 Mn(II) + H$_2$O$_2$ + 2 H$^+$ $\xrightarrow{MnP}$ 2 Mn(III) + 2 H$_2$O

*Figure 1. Suggested mechanism for CDH/MnP modification of model compounds .*Cellobiose is the main product of enzymatic cellulose degradation. ‡The electron in this step comes directly from the reduced flavin in the enzyme. CDH is known to quickly reduce this type of radicals (21). †That substances too heavy for detection with our GC/MS system have been created are supported by two facts: Firstly, new peaks were created late in the RP HPLC chromatogram. Secondly, a brown color was developed.*


GC-curve for CDH-treated model component

GC-curve for CDH- and MnP-treated model component

Mass spectrum of peak iii

Figure 2. GC/MS data from enzymatic treated model compound. Experiment performed as described in Ref 25. Peak ii corresponds to silylated, unmodified veratryl glycol. Peak i is probably incompletely silylated veratryl glycol. The mass spectrum of iii indicates that it is a demethylated version of the model compound (Figure 1). Peak iv is the didemethylated product (Figure 1). Peaks v and vi are unidentified products, likely residual carbohydrates.

We also subjected CDH treated model compound to the spectrophotometric test described in Materials and Methods. This test indicated that the CDH-treated non-phenolic model compound consumed Mn(III), *i.e.*, that the modified model compound was modified by this species (Figure 3). The non-phenolic model

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compound that had not been subjected to pretreatment with the CDH system did not consume any Mn(III), whereas a phenolic model compound gave a result similar to the CDH treated non-phenolic model compound (Figure 3). GC/MS and HPLC analysis both showed that the "new" peaks produced by the CDH treatment were consumed during the MnP treatment (Figure 2, Figure 4). We were, however, not able to identify the reaction products of this oxidation. GC/MS and HPLC analysis of non-phenolic model compound treated with the MnP system without previous CDH-treatment showed no difference to the non-treated veratryl glycol (Figure 4). In contrast to this GC/MS analysis (not shown) of the phenolic model compound treated with the MnP-system suggested that the α-carbon was oxidized to a carbonyl (Figure 1).

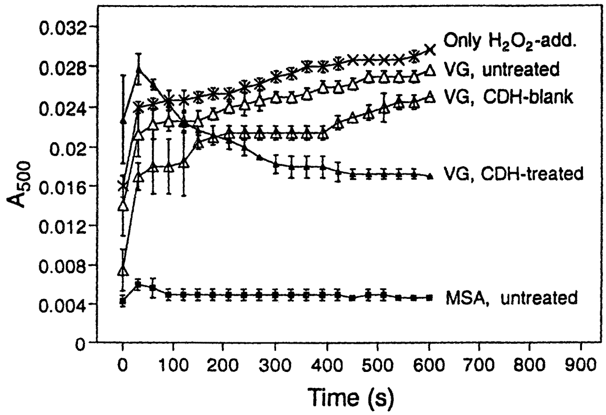

Figure 3. Spectroscopic analysis of model compound treated with the MnP system. Mn(III) oxalate has absorbance at 500 nm. It is therefore possible to determine the ability of model compounds to be oxidize by the MnP system by a spectrophotometric test (30). Data are from Ref 25.. VG, veratryl glycol; CDH-blank, treatment with ferri acetate and H_2O_2; MSA, α-methylsyringyl alcohol

In spite of that the structure of the products of the treatment with CDH followed by the MnP-system could not be identified, the results above strongly indicate that the MnP system can oxidize phenolic structures created by the CDH-system on originally non-phenolic lignin (*25*) (Figure 1). It shall be underlined that our studies are done with artificial model compounds and synthetic

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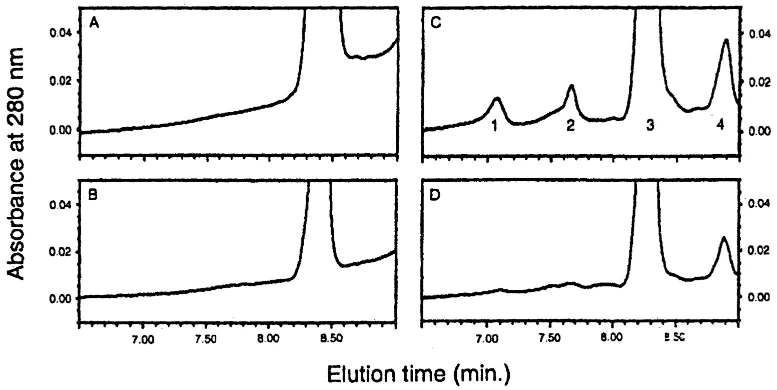

Figure 4. RP HPLC on enzymatic treated model compound. Experiment performed as described in Ref 25. A) Unmodified model compound (veratryl glycol). B) Veratryl glycol treated with the MnP system. C) Veratryl glycol treated with the CDH system. D) Veratryl glycol treated with the CDH system, and thereafter with the MnP system.

lignin instead of natural lignin. The reason is that this material is easier to analyze. Therefore the results need to be conformed on real lignin.

It is possible that the CDH system and the MnP system form an extracellular pathway in fungal lignin biodegradation, where the CDH generated hydroxyl radicals perform an initial depolymerization and introduction of phenols in non-phenolic lignins, and the Mn(III) generated by MnP oxidize these phenolic structures to allow further depolymerization. For both these enzyme systems, the active species, chelated Mn(III) in the case of MnP and chelated Fe^{2+} and hydrogen peroxide for CDH, are small and can penetrate pores in the cell wall too small for enzymes (CDH is a 89 - 111 kDa protein (*31*) and MnP have a molecular weight of approximately 43 kDa). Microscopic investigations of wood attacked by the white-rot fungus *Ceriporiopsis subvermispora* show that the structure of the woody cell wall is modified in such a way that large molecules as proteins can penetrate the cell wall (*2*). This means that in a later stage of lignin degradation direct contact between lignin and ligninases may be possible. In a recent work, Johjima et al (*32*) showed that LP

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has affinity for, and can directly oxidize lignin polymers introducing a cation radical, that can lead to various depolymerizations on lignin. Maybe LP plays a role in the degradation of condensed and other resistant lignin structures? A pathway for the different ligninases should then be as in figure 5.

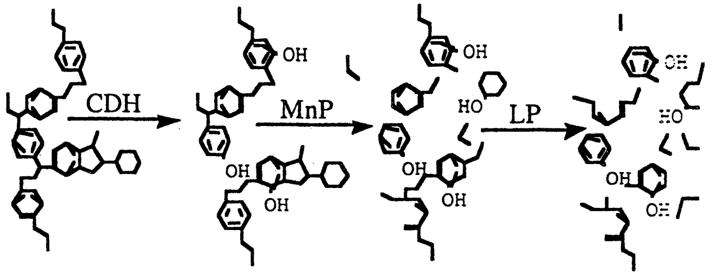

Figure 5. Suggested pathway for enzymatic lignin biodegradation. CDH performs the initial attack causing depolymerization and hydroxyl ation. The MnP system oxidizes the phenolic lignin structures giving further depolymerization. Lignin peroxidase (LP) oxidize partly soluble lignin oligomers.

MnP and CDH seem to be widely spread among white-rot fungi and might thus be important in the lignin degrading system of these basidiomycetes. Soft rot fungi and brown-rot fungi can also modify and partly degrade lignin (*5*). It is not known with certainty if these organisms produce MnP or LP, but CDH is widely produced at least by soft-rot fungi (*31,33*) and might thus be one of the enzymes responsible for this effect. The brown-rot modified lignin displays the modifications that can be expected after OH• degradation, *i.e.*, demethylations (*34*). OH• have also been detected during the growth of white- and brown-rot fungi (*35,36*).

However, whilst Kirk et al (*37*) suggested that hydroxyl radicals are not involved in lignin biodegradation by white-rot fungi, this may not be the case. This assertion was based on studies with non-phenolic model compound, that was incubated with LP or a standard Fenton's reaction (Fe^{2+} + H_2O_2). The latter gave a different type of degradation pattern than white-rot fungi.

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However, one can raise two objections against this conclusion. Firstly, a standard Fenton's reaction is not a simple OH• generating system; other reactive species than OH• such as highly oxidized iron and various activated oxygen species are produced, which can give a complex reaction pattern (*38*). Secondly, the initial reaction products formed by the OH•, *i.e.*, phenolic structures, can be oxidized by the MnP system as described above, which "hide" the traces of the hydroxyl radical (*25*,*39*).

There is interest in using ligninolytic enzymes in the pulp and paper industry as environmentally mild bleaching agents (*40*). It is reported that the presence of CDH stimulates the bleaching effect of LP and MnP on kraft pulp (*41*). This effect may be due to hydroxylations, demethoxylations and depolymerization made by hydroxyl radicals generated by CDH, that increase the solubility of the residual lignin and create attack points for Mn(II). Other explanations such as reductions of colored quinones might also be possible. CDH generated OH• may cause depolymerization of the cellulose in the pulp giving a weaker paper (*23,42*). Whether the CDH- generated hydroxyl radicals show some preference for any wood components, cellulose or lignin, is an open question. Use of CDH in bleaching probably requires anyway a careful balance of the dosage in order to obtain the desired effect without too much loss of paper properties.

Acknowledgment's The authors thank Josef Gierer for fruitful discussions and the School of Chemistry and Chemical Engineering at Royal Institute of Technology for economical support.

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Chapter 30

Treatment of Poplar High-Yield Pulp with Fungal Peroxidases: From Laboratory to Pilot Scale Study

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The objective of this European project (AIR3-CT94-2065) was to improve the quality of an industrial poplar high-yield pulp by introducing an treatment with a crude enzyme preparation enriched in the ligninolytic manganese peroxidases (MnP). The enzymes used in this study were produced from an hypersecretory strain of *Phanerochaete chrysosporium*. Optimizing culture conditions and medium

In Oxidative Delignification Chemistry; Argyropoulos, D.;
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composition led to a production of 7200 Units/l MnP and 2100 Units/l lignin peroxidases (LiP) in a 100 liter bioreactor. Enzyme availability allowed pulp treatment from the laboratory scale up to a 100 kg pilot scale. After optimization, the treatment with crude MnP at the laboratory scale led to an energy saving of up to 25% during secondary refining with PFI mill with a slight improvement in some of the pulp properties such as tensile index, RBA index or breaking length. This effect could be attributed either to lignin modification and fibrillation of the microfibrils, as shown by chemical analysis and transmission electron microscopy. This enzymatic refining catalyzed by the crude MnP was further confirmed at a ~100 kg scale on industrial poplar alkaline-peroxide pulp.

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Introduction

The use of white rot fungi and their enzymes as aids in bleaching and fiber quality improvement was proposed more than 15 years ago.

The introduction of enzymes in the kraft pulp bleaching process was carried out for the first time in 1986 with xylanases *(1)*. Pulp treatment before the chlorine dioxide stage saves about 20 % of the bleaching chemicals. This is mostly due to leaching of lignin fragments together with the hydrolyzed xylan fragments released by the enzyme *(1,2)*. Today, several pulp mills regularly use hemicellulases in their process for various purposes. An important factor in this success was the simultaneous research in enzyme based technology and in enzyme production in high yields suitable for industrial applications *(1 ,3)*.

The possibility of using ligninolytic enzymes in the pulp and paper industry was also proposed after their discovery in 1983 *(4,5)*. These enzymes are able to depolymerize lignin *in vitro* and to catalyze the main reactions involved in lignin degradation by the whole fungus, such as cleavage of the alkyl aryl ether bonds of the polymer.

Lignin peroxidase (LiP) was the first enzyme of the ligninolytic system of *Phanerochaete chrysosporium* to be discovered. However LiP was found to be a poor candidate for fiber delignification and bleach boosting, as compared to xylanase. This was partly related to the poor diffusion of the enzyme inside the fiber cell wall, its high sensitivity to H_2O_2 in the absence of a protecting agent (veratryl alcohol), and to the formation of conjugated carbonyls and quinone compounds during lignin oxidation which have a strong negative impact on the brightness of pulps *(6,7)*.

Manganese peroxidase, a second type of enzyme discovered in 1984, seemed to be more promising as it was demonstrated to be essential in kraft pulp bleaching by white rot fungi *(8)*. Its catalytic action is related to the formation of MnIII-chelates in the presence of hydrogen peroxide, manganese II and organic acids like malonate or oxalate. The Mn oxidants formed are able to diffuse and penetrate into the pulps to degrade lignin at a distance from the enzyme. Delignification and significant increase of brightness were indeed reported when isolated MnP was incubated in suitable conditions with kraft pulps *(8,9)*.

The last ligninolytic enzyme tested so far is laccase. As for MnP, the presence of a mediator able to promote oxidation at a distance from the enzyme is a requisite for delignification and fiber property improvement *(10,11,12,13)*. Currently, the combination of laccase with an organic mediator is developed at the pilot stage and is probably the lignin-oxidizing system closest to application in the pulp and paper industry *(3,13)*.

In this context, the objective of our European project was to study an enzymatic treatment based mainly on MnP catalysis and to demonstrate its positive impact on pulp properties up to a 100 kg pilot scale on an industrial alkaline peroxide mechanical pulp.

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Experimental

Enzyme production

Phanerochaete chrysosporium I-1512 (Institut Pasteur, Paris, France) was selected by INRA (LBCF) for its ability to secrete high level of enzymes in defined conditions, and was used throughout this study *(14)*. Culture conditions in a 2.5 liter fermentor and scale up to 100 liter bioreactor were performed as described in ref *(15)*. The crude enzyme preparation was a concentrate of culture fluids obtained by ultrafiltration *(16)*.

Lignin peroxidase (LiP) and manganese peroxidase (MnP) activities in cultures and concentrates were monitored according to the published procedures by using veratryl alcohol and vanillyl acetone as substrates, respectively *(17,18)*.

Wood Pulp

The alkaline peroxide mechanical market pulp used was produced by Sicem Saga S.p.A. (Italy). The wood was reduced into big matches in a refiner equipped with special customized plates before impregnation with alkaline peroxide. After chemical reaction, the wood matches were primary

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and secondary refined, then screened before balling. The pulps used for enzymatic treatments were always secondary refined.

Lignin Analysis

The lignin content in β-O-4 linked units was determined on the treated pulps by the thioacidolysis method as described by Lapierre *et al (19)*. The degradation yields of Guaiacyl (G) and Syringyl (S) monomers were determined relative to their content in the non-oxidized sample *(20)*.

Electron microscopy

Pulps were observed by transmission electron microscopy (TEM) on ultrathin sections embedded in resin. The samples were contrasted with uranyl acetate or stained for polysaccharides with the periodic acid-silver reagent of Thiery adapted by Ruel *(21)*. Immunolabelling of MnP in pulps was performed with polyclonal antibodies revealed with a gold-protein A conjugate as previously reported *(22)*.

Pulps were also observed by scanning electron microscopy (SEM) after dehydration by critical point drying *(23)*.

Enzymatic treatment of pulp

Unfractionated concentrated crude enzyme preparations containing MnP were used throughout this study. The LiP to MnP ratios in the preparations were around 0.16 for enzymes coming from a 2,5 liter bioreactor and reached a value of 0.29 for enzymes from 100 liter fermentors. No cellulases nor hemicellulases activities were found in the concentrated culture fluids.

The secondary refined poplar alkaline peroxide pulp (APP) was treated at 5 and 10% consistency within sealed plastic bags after mixing of the following components: 100 mM lactate buffer pH 4.5, 1 mM of $MnSO_4$, 15 or 25 U MnP/g of dry pulp. The hydrogen peroxide was mixed with the pulp at a final concentration of 0.01, 0. 1 or 1 mM, just before sealing the bag. Incubation was performed for 1 or 2 hours at 40°C in a thermostated bath.

The enzymatic treatment was followed by an acidic wash (20 min at pH 2.5 at 2% consistency), a chelating treatment (0.4% w/w DTPA, 25 min at 60°C, at 4% consistency) and peroxide bleaching stage (1% H_2O_2 w/w, 1.5% NaOH, 4% Na_2SiO_3, 240 min, 60°C, 16% consistency).

For pilot scale trials, 80 to 100 kg of APP pulp were treated at 25°C with 1,200,000 to 1,500, 000 Units MnP at 10% consistency in the presence of 1 mM $MnSO_4$ and 0.1 mM peroxide. Reaction time was decreased to 1.5 hour reaction. The pulps were then washed and bleached as described above

Pulp quality evaluation

The physical and optical properties were evaluated on handsheets prepared according to ISO 5269-2 method. Tensile (TS), breaking length (BL), stretch (S), burst (B), tear (T), opacity (O) and brightness (BT) indexes were determined according to NF-EN-ISO1924-2 (TS, BL, S), NF-Q-03053 (B), NF-EN-21974 (T), NF-Q-03040 (O), ISO-2470 (BT) standards. Morphological characteristics of pulps were determined by analysis with PQM 1000 and CyberMetrix analyzers.

Results and Discussion

Production of crude MnP

The immobilized cell bioreactor was shown to be suitable for high enzyme level production at 100 liter scale *(15)*. Under these cultivation conditions, *Phanerochaete chrysosporium* I 1512 was able to produce up to 7200 U/l within 160 hours in 100 l nominal volume. LiP was also produced, although at a lower yield (~ 2100 U/l), but no cellulases nor hemicellulases were found in the culture fluid.

Treatment of poplar alkaline peroxide pulps (APP) at laboratory scale

Two critical parameters for MnP catalysis are the poor stability of the enzyme during the reaction in the presence of excess peroxide and the formation of the MnIII chelates followed by their diffusion into the reaction bulk and the fibers. For these reasons, the incubation was performed in sealed bags where pulps at 5 or 10% consistency were first impregnated with manganese and low level of hydrogen peroxide before mixing with enzymes. After bleaching, the pulp characteristics were different in reference and crude-MnP treated samples, as shown in **table I** for the 5% consistency trial.

The first effect evidenced was a loss of brightness, which could be partially compensated for by acidic washing and peroxide bleaching **(table I)**. Nevertheless, 8 points in brightness could not be recovered, compared to reference bleached pulp. This is probably due to manganese deposit on fibers, not fully removed by acid washing and the chelation step *(24)*, or to carbonyls and quinones formation, as described for LiP catalysis *(6,7)*. The other pulp characteristics remained identical, with a slight improvement of tensile index and breaking length of pulps after treatment with crude MnP, but to the detriment of the tear index.

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Table I. Properties of bleached APP pulp after enzyme treatments at laboratory scale (5% consistency)

	reference pulp		pulp treated with crude MnP		
MnP content U/ g	0	0	25	25	25
Peroxide[a/], mM	0	0	1	0.1	0.01
Post-reaction sequence [b/]	no	A-C-P	A-C-P	A-C-P	A-C-P
Freeness, ml CSF	80	70	57	60	57
Brightness, % ISO	74.4	79.4	72.5	72.4	71.5
Opacity, %	66.7	66.9	65.7	67	65.6
Tensile index, Nm/g	62.9	66.8	67.9	68.5	70.6
Tear index, mNm^2/g	3.31	3.22	3.57	2.81	3.01
Breaking length, m	6350	6810	6920	6980	7190
Fiber length, mm	0.89	0.93	0.89	0.86	0.85
Fiber width, μm	26.6	34.1	27.7	28.8	29.2
Coarseness, mg/m	0.153	0.098	0.147	0.141	0.137
RBA index, %	nd[c/]	45.7	43.8	48	44.2
Shive content, %	0.02	0.04	0.06	0.05	0.05

[a/] during catalysis ; [b/] A : acidic washing ; C : chelating stage ; P : peroxide bleaching stage ; [c/]nd : not determined

In fact, the main impact of enzymatic treatment was either on the final lower freeness of the pulps, indicating a direct refining of the fibers and on the energy required to refine the pulp at a given freeness without loss of pulp properties (**figure 1**). Indeed, when pulp were treated at 5% consistency, as much as 25% energy can be saved to reach a freeness of 90ml CSF, compared to reference bleached pulp (**figure 1**).

Considering the chemical and structural characteristics of bleached pulps, lignin was oxidized by the enzymes and/or removed during the peroxide bleaching sequence. Indeed, about 10 to 15% of β-O-4 linked G and S units from lignins were lost after the whole fiber processing including MnP treatment (5% consistency, 25U MnP/g ; 0.01mM peroxide), compared to reference pulps.

Characterization of the pulps by Scanning Electron Microscopy (SEM) revealed furthermore that MnP induced a separation of the fibrils which were clearly individualized at the surface of the fiber (**figure 2 A and B**). Transmission Electron Microscopy (TEM) observations revealed also that MnP penetrated in the fiber wall (**figure 3**) and induced an internal fibrillation (data not shown). This resulted in the individualization of microfibrils. The comparison of the three different peroxide concentrations during the treatment indicated that the intermediate concentration (0.1 mM) at 5% pulp consistency resulted in deeper internal alterations in the fiber walls and a more intense loosening of the secondary wall (data not shown).

When pulp consistency in reaction medium increased from 5 to 10%, the same general effects of crude MnP on pulp properties were observed, with a final brightness almost fully recovered after bleaching (only 2 points lower

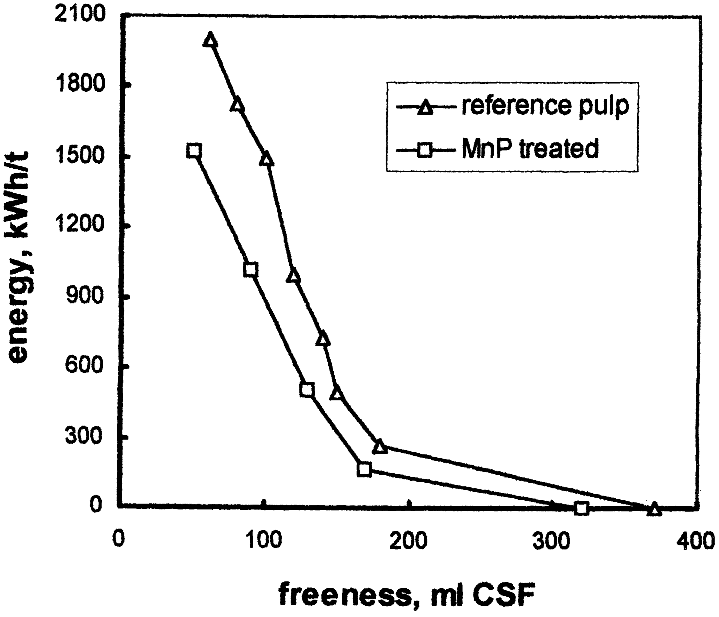

figure 1. Energy requirement for the secondary refining of bleached reference and crude-MnP-treated pulps (5% consistency, 25U MnP/g, 0.1mM H_2O_2)

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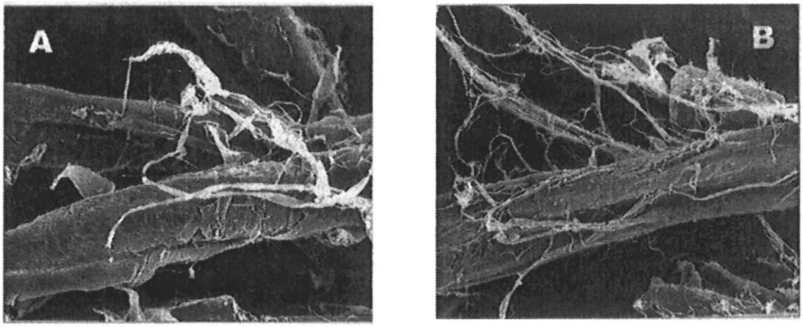

figure 2. Surface fibrillation after bleaching ; A : reference pulp ; B : pulp treated by crude-MnP before bleaching.

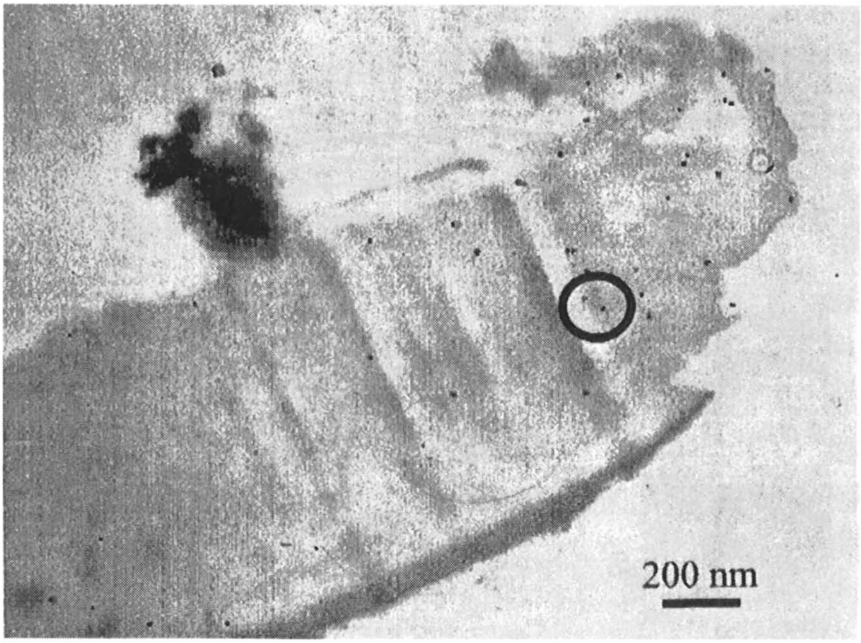

figure 3. Penetration of MnP in pulps detected by immunolabelling of the enzyme. The diameter of gold particle is 5 nm and has been enhanced with silver.

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than the bleached reference pulp). Also, the improvement of beating ability in PFI mill was confirmed after crude-MnP treatment (data not shown).

In order to prepare the pilot plant trial, an experiment was carried out at laboratory scale with 15 U of MnP per g of pulp (10% consistency, 1mM $MnSO_4$, 100mM lactate buffer, 0.1mM H_2O_2, 1.5 hour time reaction). Bleached reference and MnP-treated pulps were beaten in a PFI mill with the same energy at 3000 rpm to evaluate beating behavior and strength development (**table II**).

As expected from the previous experiments carried out with 25 U of crude MnP, enzyme treatment modified mainly the fiber refining behavior, while strength development was only slightly improved. For the same energy applied in the PFI mill, the MnP-treated pulp was more rapidly beaten with lower freeness reached (100 ml CSF) compared to the reference pulp (130 ml CSF, **table II**). Tensile strength was slightly improved while the tear index was decreased, probably due to some fiber structure degradation related to the internal fibrillations evidenced in other trials (see above). Again, treatment with crude MnP induced some loss of brightness, which was here fully recovered after peroxide post-bleaching and beating. The brightness gain was however obtained with lower peroxide consumption (12 points of brightness gain per % of consumed peroxide), indicating that chromophores involved could be partially and more easily removed during the bleaching of pulps.

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Table II. Properties of enzyme treated bleached APP pulps after refining in PFI mill

Pulp grade	Reference	MnP-treated
Initial brightness[a/], % ISO	81.3	nd[c/]
Brightness after acidic wash, % ISO	82.2	77.3
Final brightness[b/], % ISO	81.1	80.9
% consumed H_2O_2	40	30
Freeness, ml CSF	130	100
Tensile index, Nm/g	56.2	57.7
Tear index, mNm^2/g	2.83	2.64
Fiber length, mm	0.90	0.91
Fiber width, μm	34.3	32.2
Fiber coarseness, mg/m	0,101	0.106
Shive content, %	0.10	0.10

[a/] before bleaching step ; [b/] after bleaching and beating ; [c/] nd : not determined

Pilot plant scale experiments

Two large scale trials were performed at the bleaching pilot plant from the Centre Technique du Papier (Grenoble). Eighty to hundred kilograms of industrial pulp with high and low initial freeness were treated with 15U crude MnP/ g of pulp in the conditions defined at laboratory scale -see above.

The industrial pulp with initial freeness of 160 was used in the first test. As shown for laboratory scale experiments, pulp brightness was not significantly improved by the MnP and peroxide treatments (**table III**). However, enzymatic refining was confirmed when batches (MnP-treated and reference) were beaten at 4 % consistency in a semi-industrial conical Conflo refiner as used by paper mills for preparing pulps for paper manufacture. Indeed, the mechanical treatment applied on the bleached pulps induced a freeness shift from 145 to 80 ml CSF with 20.9 kWh/t energy consumption whereas the MnP treated pulps needed only 19.2 kWh/t to shift from 138 to 74 ml CSF, corresponding to an energy saving of about 7%. This value was lower than that obtained previously on a PFI mill at the laboratory scale, probably due to the higher refined state of the initial pulp at the pilot scale (160 ml CSF, versus 370 for the laboratory scale study) and to the technical differences between Conflo and PFI refiner, having each a specific mechanical action on fibers which is not directly comparable.

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Table III. Properties of the enzyme-treated pulps after bleaching at pilot plant scale.

	Reference pulp		MnP-treated pulp		
	Initial pulp	bleached pulp	After crude MnP	After acidic wash	bleached pulp
Freeness, ml	160	145	170	160	138
Brighthness, % ISO	71.2	70.8	69.7	68.1	71.3
Tensile index, Nm/g	35.3	42.8	34.1	38.3	41.8
Breaking length, m	3590	4370	3480	3900	4270
Tear index, mNm^2/g	2.93	3.25	2.96	3.00	3.04

Transmission electron microscopy further confirmed the impact of the crude MnP preparation on morphological features of pulps. The most conspicuous effect of the enzyme treatment at pilot plant scale was the significant external and internal fibrillation of S2 layer (**figure 4**) as evidenced at laboratory scale. Also, catalysis of crude MnP on lignin at this scale and under this experimental condition exhibited an unusual feature. Indeed, the proportion of lignin fraction in pulps sensitive to thioacidolysis depolymerization increased by 20%, suggesting that some intermonomeric lignin-lignin bonds or intermolecular lignin-polysaccharides bonds were cleaved *(19,25)*.

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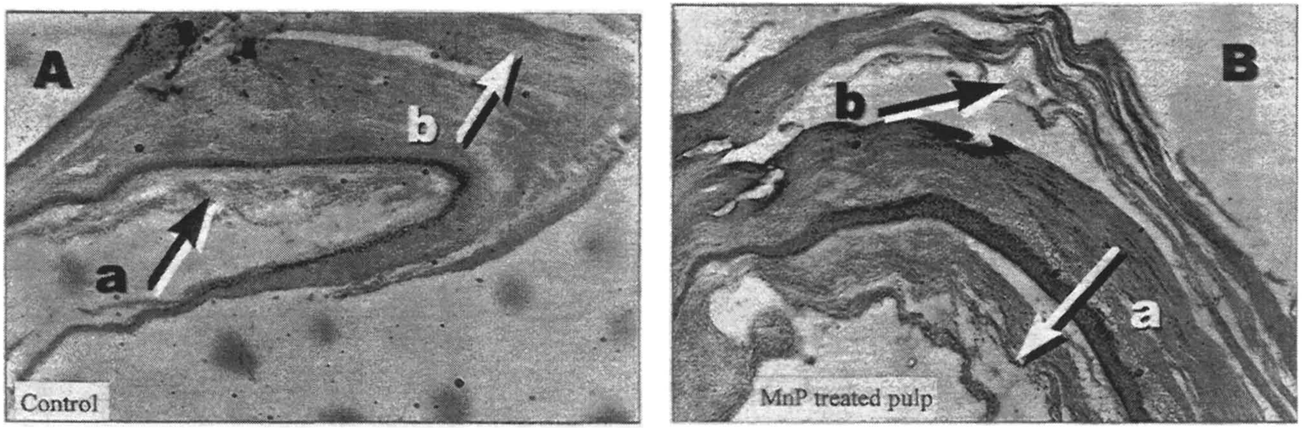

figure 4. Microfibrils separation at the internal (arrow a) and external parts (arrow b) of S2 layer in reference (A) and pulp treated at pilot plant scale (B).

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When pulps of lower freeness were used (246 ml CSF after bleaching for reference pulp), the energy consumption in the Conflo refiner to reach a freeness of ~90 was similar for reference and crude-MnP treated samples (data not shown). However, reference pulps could not reach freeness lower than 83 ml CSF, despite higher energy applied whereas the MnP-treated pulp could be beaten further to higher freeness (data not shown). Also, an increase in the tensile index of 10 % was obtained for MnP treated pulps (55 Nm/g) refined at 96 ml CSF, compared to the reference pulp (50.6 Nm/g) beaten at 98 ml CSF. Thus, easier refining with some improvement in fiber properties was demonstrated, although the action of crude MnP was more pronounced on more refined pulp with higher freeness.

CONCLUSIONS

The use of a crude manganese peroxidases preparation containing residual lignin peroxidase activity on a market poplar high yield-pulp (APP) was optimized to improve pulp quality and beating behavior. The results showed that the main effect of crude MnP at laboratory scale was an enzymatic refining that facilitated fibrillation and reduced the energy consumption significantly during beating. Enzyme availability at a high scale confirmed these results on 100kg of pulp batches. However, energy saving during beating was lower at the pilot scale, in a process closer to industrial conditions. In all cases, fiber properties were not negatively affected, with even slight improvements of some characteristics. Further studies are needed to optimize pulp treatments in order to boost the enzymatic refining of fibers and enhance their properties.

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Chapter 31

Designing a Manganese Peroxidase

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Manganese peroxidase (MnP) from the white rot fungus *Phanerochaete chrysosporium* plays a vital role in lignin degradation. MnP functions by oxidizing Mn(II) to Mn(III) which serves as a diffusible oxidant. To test the current understanding of the structure and function of MnP and to find an alternative catalyst for oxidative delignification, we employed a new approach involving the design and engineering of a MnP using cytochrome *c* peroxidase (C*c*P) from bakers' yeast. Based on structural comparisons and computer modeling, we created a C*c*P mutant (MnC*c*P) that binds Mn(II) in a manner similar to the native enzyme and showed that the incorporation of the Mn(II)-binding site facilitates Mn(II) oxidation. Further mutations of key active site residues (W191 and W51) in MnC*c*P to the corresponding Phe in MnP conferred even more MnP activity of our protein model. The two mutations do not contribute equally to the activity increase. A much larger increase arises from the W51F mutation because W51 stabilizes compound II. Since the reaction of compound II with substrate is rate-limiting, a more reactive compound II increases MnP activity. Thus our approach is also capable of offering new insight into the structure↔function relationships of MnP and C*c*P.

Lignin is a complex phenylpropanoid polymer comprising 15-30% of woody plant cell wall materials. It gives plants their rigidity and provides protection from microbial attack. Lignin is the second most abundant biopolymer (after cellulose) on earth and is the most abundant renewable aromatic material. Therefore, controlled delignification is important not only for the global carbon cycle, but also for industrial applications such as wood and straw pulping, pulp bleaching, and conversion of lignin to higher value products.

Manganese peroxidase (MnP) from the white-rot basidiomycete *Phanerochaete chrysosporium*, together with lignin peroxidase (LiP) and an H_2O_2-generating system, plays a key role in the oxidative delignification process (*1-3*). This white-rot fungus is one of the few fungi known to degrade lignin extensively. In addition, MnP is also involved in biodegradation of many aromatic pollutants (*4-6*). Thus understanding the structure and function of MnP has been the focus of extensive research. For example, biochemical and spectroscopic studies have established the reaction mechanism and pointed to the importance of Mn(II) oxidation in the delignification process (*2,3,7-10*). The MnP crystal structure (*11*) provided a structural basis for the biochemical and spectroscopic studies. The development of recombinant MnP and its protein expression systems (*12-15*) allowed the use of site-directed and random mutagenesis to probe the role of each amino acid residue in the structure and function of MnP (*16-19*).

Tremendous progress towards understanding MnP has been made but industrial applications have not been realized due to the high cost and low catalyst stability. Consequently, several groups have focused on the use of biomimetic synthetic models such as small, stable, organic/inorganic molecules that mimic the essence of MnP (*20,21*). However, the biomimetic models have not been able to mimic the full features of MnP, including catalytic turnovers.

Our group is interested in an experimental approach that combines the advantages of native enzyme and biomimetic model system studies. We use small, stable, easy-to-produce and well-characterized proteins as templates for the construction of protein models of MnP and other proteins. Like native enzymes, protein models are comprised of the twenty amino acids and therefore a protein-based model should more closely mimic MnP and support catalytic turnover. As with synthetic models, the protein models can be made more cost-effectively than the native system.

We chose cytochrome *c* peroxidase (C*c*P) as the template for our studies. C*c*P is a heme peroxidase from *Saccharomyces cerevisiae* (*22*) and, along with MnP, is a member of the plant peroxidase superfamily (*23*). C*c*P catalyzes the oxidation of ferrocytochrome *c* and is not capable of supporting delignification. Therefore, it is an interesting challenge to evolve this bakers' yeast enzyme into

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an enzyme capable of MnP activity. In this way, we aim to develop a new and cheaper catalyst for delignification.

Our work is aided greatly by comprehensive studies of native C*c*P (*24-32*). The engineering, expression, and purification of C*c*P have been optimized and the high yields (>100 mg per L of culture) of pure C*c*P obtained will facilitate our biomimetic studies.

Perhaps the biggest advantage of redesigning C*c*P into a MnP is the ability to identify the minimum of structural features required for MnP function. Since C*c*P displays little MnP activity, the introduction of important structural features from MnP into C*c*P allows elucidation of the relative importance of those features. Thereby, this approach can serve as a touchstone whereby the knowledge about the structure and function of MnP and C*c*P is tested. More importantly, this bottom-up approach may reveal important structural features and mechanistic insights that are otherwise difficult to identify thereby providing information complementary to that obtained from the study of the native MnP system.

In this chapter, we will provide an overview of our general strategy for creating a MnP based on C*c*P, focusing on the role of the Mn(II)-binding site and the lack of active site tryptophans on peroxidase activity. Spectroscopic studies and MnP activity assay results of the designed enzymes will be presented to illustrate how each structural element affects the structure and function of our MnP model. The ability of this approach to reveal new information not readily available from the study of native enzymes or synthetic models will also be demonstrated.

Structural Comparison of C*c*P and MnP

The crystal structures of C*c*P and MnP are shown in Figure 1. Despite the limited sequence homology of <20% between the two peroxidases, the overall structural folds of the two enzymes are quite similar. Excluding the carbohydrate-binding domain on the bottom of the MnP structure shown in Figure 1, the protein backbones superimpose quite well (*11*). C*c*P and MnP form one of countless examples in biology where nature utilizes the same thermodynamically stable scaffold but widely different sequences to achieve diversity of function. This observation forms the basis of our protein redesign approach. One of the goals of our research is to determine how nature utilizes different sequences in the same scaffold.

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The active site structures of C*c*P and MnP are shown in Figure 2. Residues important for the enzymes' reaction with hydrogen peroxide (e.g., proximal and distal histidines, proximal aspartate and distal arginine) are conserved between C*c*P and MnP (and other members of the superfamily) (*23*). Even the distal hydrogen-bonding network is conserved between the two enzymes. Despite these similarities, the native substrate for C*c*P is ferrocytochrome *c* and the enzyme possesses negligible MnP activity.

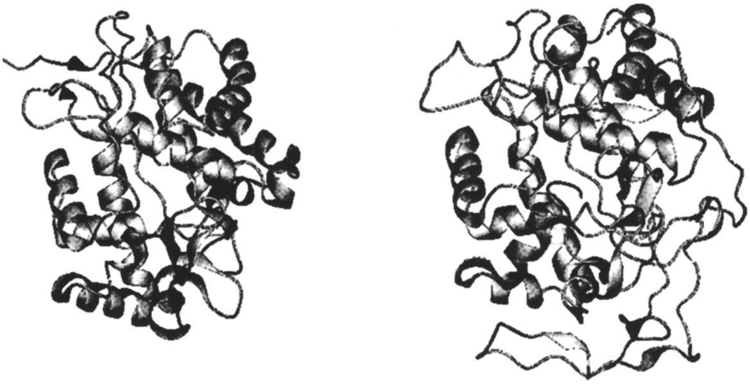

Figure 1. The tertiary structures of C*c*P (left) and MnP (right).

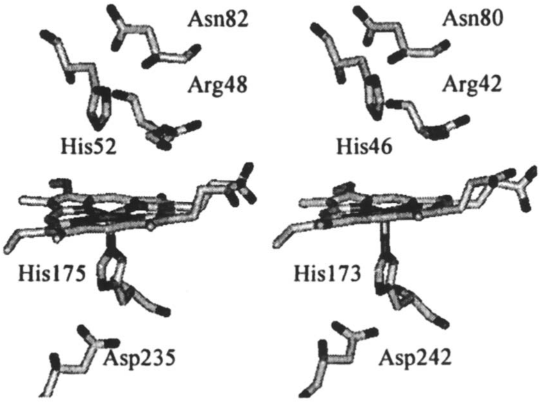

Figure 2. The similarity of the active sites between C*c*P (left) and MnP (right).

Subtle structural differences must play a major role in determining the functions of these two enzymes. The key structural differences between C*c*P and MnP are shown in Figure 3. First, C*c*P lacks the Mn(II)-binding site found in MnP. The Mn(II)-binding site was proposed based on the X-ray structure of

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MnP (*11*). Kinetic studies of MnP and its variants containing mutations of the proposed Mn(II)-binding site ligands confirmed their role in binding Mn(II) and supporting MnP activity (*16,17,19,33*). Therefore, the lack of a Mn(II)-binding site in C*c*P may prevent efficient oxidation of Mn(II) to Mn(III), a key reaction in the delignification process.

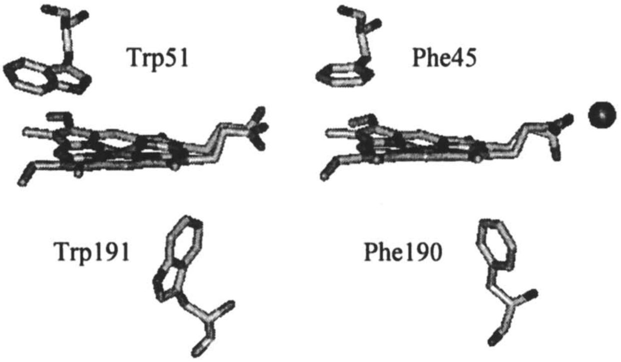

Figure 3. Major differences of the active sites between C*c*P (left) and MnP (right).

The second structural difference is that the heme cavity of C*c*P contains two tryptophans while MnP has phenylalanines at the corresponding positions. This structural difference results in mechanistic differences between the two enzymes. As shown in scheme 1, the overall catalytic cycles are similar but the nature of the first high-valent intermediate varies and the ability to oxidize Mn(II) to Mn(III) differs between the two enzymes. Because C*c*P possesses the more readily oxidized residue tryptophan, the stable compound I′ contains a ferryl (Fe(IV)=O) and a Trp radical. By contrast, the presence of Phe in MnP precludes formation of a protein radical at that position and MnP compound I contains a ferryl and porphyrin π cation radical.

$$\mathrm{Fe(III)} + \mathrm{H_2O_2} \rightarrow \mathrm{Fe(IV)\text{-}oxo,\ Trp{+}\bullet} + \mathrm{H_2O}$$
$$\mathrm{Fe(IV)\text{-}oxo} + Cc^{2+} \rightarrow \mathrm{Fe(IV)\text{-}oxo} + Cc^{3+}$$
$$\mathrm{Fe(IV)\text{-}oxo} + 2\mathrm{H^+} + Cc^{2+} \rightarrow \mathrm{Fe(III)} + Cc^{3+} + \mathrm{H_2O}$$

$$\mathrm{Fe(III)} + \mathrm{H_2O_2} \rightarrow \mathrm{Fe(IV)\text{-}oxo,\ Por{+}\bullet}$$
$$\mathrm{Fe(IV)\text{-}oxo} + \mathrm{Mn(II)} \rightarrow \mathrm{Fe(IV)\text{-}oxo} + \mathrm{Mn(III)}$$
$$\mathrm{Fe(IV)\text{-}oxo} + 2\mathrm{H^+} + \mathrm{Mn(II)} \rightarrow \mathrm{Fe(III)} + \mathrm{Mn(III)} + \mathrm{H_2O}$$

Scheme 1. Catalytic cycles of C*c*P (top) and MnP (bottom).

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Design of a Mn(II)-Binding Site into C*c*P

The goal of our research is to design a Mn(II)-binding site into C*c*P and determine the effect of replacing the active site tryptophans with phenylalanines. We designed the Mn(II)-binding site first. The design of a Mn(II)-binding site in C*c*P was based on a comparison of the sequences and crystal structures of MnP and C*c*P (see Figure 4) The putative Mn(II)-binding site contains a manganese ion in a pseudo octahedral environment formed by three carboxylates, two water molecules and a heme propionate. The proposed ligands for Mn(II) in MnP are E35, E39 and D179 and the corresponding residues in C*c*P are G41, V45 and H181. In order to create an analogous site in C*c*P, we introduced the G41E, V45E, and H181D mutations into C*c*P. The resulting mutant is called MnC*c*P in this chapter and in our previous publications (*34-36*). Similar work has been described by Wilcox et al. (*37*) who engineered Mn(II)-binding site in C*c*P at a location similar to ours (*34*).

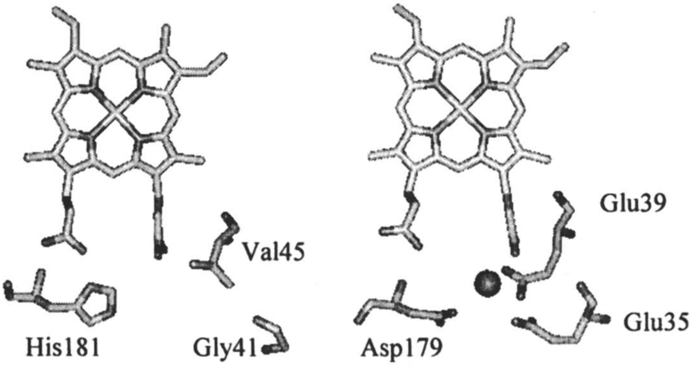

Figure 4. Mn(II)-binding site in MnP (right) and the corresponding region in C*c*P (left).

MnC*c*P was expressed in *E. coli* and purified to homogeneity (*34*). The UV-visible and paramagnetically shifted proton NMR spectra for WTC*c*P and MnC*c*P at pH 6 were similar and consistent with the presence of high-spin ferric heme. Addition of hydrogen peroxide to samples of MnC*c*P converts the enzyme to compound I′ (ferryl, tryptophan radical). The conversion is dependent on the concentration of peroxide and a second order rate constant of 10^7 M^{-1} s^{-1} was determined from a plot of rate versus hydrogen peroxide concentration (*34*). The rate is equal to the rate observed for WT within experimental error. These results suggest that the mutations used to create a Mn(II)-binding site in C*c*P do not adversely affect the ability of the enzyme to react with hydrogen peroxide.

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The binding of Mn(II) to WT and MnC*c*P was studied by electronic absorption and paramagnetically-shifted proton NMR spectroscopies (*34,35*). The addition of 100eq of Mn(II) to WT did not result in detectable spectral changes. On the other hand, changes in the heme Soret region are observed upon addition of Mn(II) to solutions of MnC*c*P and the spectral changes are more readily observed in difference spectra (Mn(II)MnC*c*P – MnC*c*P). The difference spectra observed for MnC*c*P are similar to those reported for native MnP (*7*). The spectral changes that occur upon Mn(II) addition indicate a specific interaction between the heme and Mn(II). A Hill plot and a double reciprocal plot of the data for native MnP revealed the presence of a single Mn(II)-binding site with a K_D of 9.6 μM. Similar treatment of our data for MnC*c*P showed that a single Mn(II)-binding site with an apparent dissociation constant (K_D) of 125.5 μM was created in MnC*c*P.

In order to confirm the location of the Mn(II)-binding site in MnC*c*P, the binding of Mn(II) to MnC*c*P was studied by paramagnetically shifted proton NMR spectroscopy (*35*). Cyanide derivatives were used for these studies because of the favorable properties of the low-spin cyanide adducts. While the spectra for WTC*c*PCN and MnC*c*PCN are similar, the behavior with respect to manganese addition differs. The addition of Mn(II) to a sample of WTC*c*PCN had no effect on the paramagnetic NMR spectrum. On the other hand, the addition of Mn(II) to the cyanide adduct of MnC*c*P resulted in the broadening and eventual loss of four peaks in the paramagnetic region. The broadened resonances are assigned to R48, the heme 7-propionate α-proton and β-protons based on a complete assignment of paramagnetically shifted resonances using one- and two-dimensional experiments. The selective broadening of these resonances locates the Mn(II) near its designed site (Figure 4) since the unpaired electrons on Mn(II) can broaden the NMR signals of protons close to Mn(II) (*9*).

The effect of the engineered Mn(II)-binding site on the MnP activity of MnC*c*P was determined by measuring the steady-state oxidation of Mn(II) (*34*). Assays were conducted in 50 mM malonate, pH 6, and the steady-state oxidation of Mn(II) was determined by measuring the formation of the Mn(III)-malonate complex at 270 nm (*12*). Under these conditions, the rate of Mn(II) oxidation catalyzed by MnC*c*P was at least fivefold greater than that observed for WT. These results provide strong evidence for the pivotal role of the Mn(II)-binding site in supporting MnP activity.

The Role of Tryptophans in Peroxidase Activities

The incorporation of the G41E, V45E, and H181D mutations into C*c*P provided a model through which the importance of other structural features could be evaluated. The active site of C*c*P contains two tryptophan residues (at positions 51 and 191) while the corresponding residues in MnP are phenylalanines (see Figure 3.) Throughout the peroxidase superfamily, phenylalanines are more commonly found at these positions. The presence of W191 allows C*c*P to utilize a unique catalytic cycle as it functions as the electron transfer partner of cytochrome *c* (*22,28*). For typical peroxidases such as MnP, the organic radical in compound I resides on the porphyrin. Erman et al had previously shown that the W191F mutation in C*c*P led to the transient formation of a porphyrin π-cation radical (*38*). Since W51 and W191 are located close to the heme pocket and Mn(II) oxidation is coupled to the high-valent intermediates, we surmised that their presence may influence not only the location of the radical, but also Mn(II) oxidation activity.

To investigate the effect of the W-to-F mutations on the location of the radical, we constructed and purified C*c*P(W191F) and C*c*P(W51F, W191F) (*36*). A stopped-flow study of hydrogen peroxide activity showed that the W191F mutation resulted in the appearance of compound I (ferryl and porphyrin π-cation radical) with an observable life-time (decay rate: 60 s^{-1}) that is similar to that reported by Erman et al. (*38*). We further demonstrated that the W191F/W51F double mutations increased the lifetime of compound I (decay rate 18 s^{-1}). The spectra are shown in Figure 5. These results suggest that the absence of redox active tryptophan residues around the heme pocket is a primary

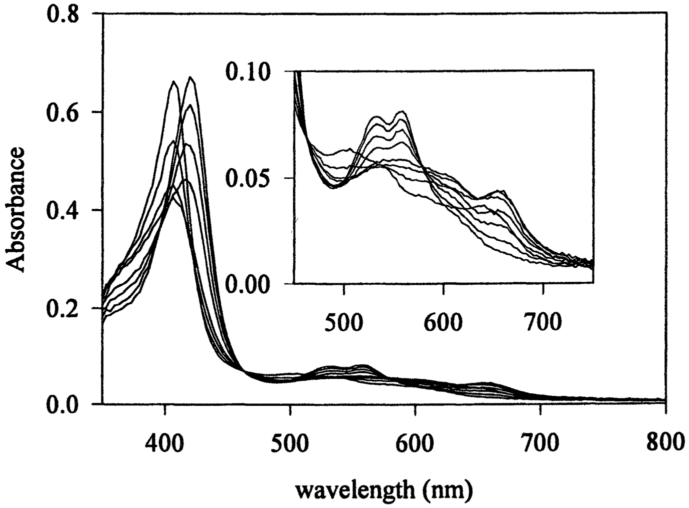

Figure 5. UV-Visible spectra for the reaction of C*c*P(W51F, W191F) with hydrogen peroxide.

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means for heme peroxidases to generate compound I and that the effects of W-to-F mutations are additive.

After the examination of W-to-F mutations on the location of the radical, we turned our attention to the effect of these mutations on MnP activity, specifically the ability to oxidize Mn(II). Toward this end, we constructed and purified MnC*c*P(W191F), MnC*c*P(W51F) and MnC*c*P(W51F, W191F) (*36*). These mutants build on MnC*c*P and replace the proximal, distal, and both active site trytophans with phenylalanine as found in native MnP. A steady-state kinetic study of manganese oxidation was performed at pH 5 in 500 mM malonate. The Lineweaver-Burk plots for MnC*c*P, MnC*c*P(W191F), MnC*c*P(W51F) and MnC*c*P(W51F, W191F) are shown in Figure 6. The parameters are compiled in Table 1.

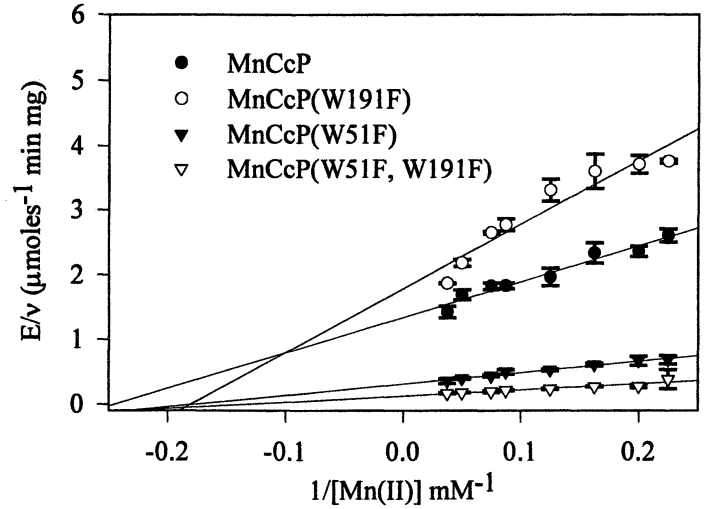

Figure 6. Lineweaver-Burk Plots for MnC*c*P (black circle), MnC*c*P(W191F) (white circle), MnC*c*P (W51F) (white triangle), and MnC*c*P (W51F, W191F) (black triangle).

As can be seen from the plots, introduction of the W191F mutation to MnC*c*P did not improve MnP activity (V_{max}, MnC*c*P: 0.750 μmoles min^{-1} mg^{-1}; MnC*c*P(W191F): 0.560 μmoles min^{-1} mg^{-1}) despite the fact that introduction of the same mutation to C*c*P formed a transient compound I (decay rate: 60 s^{-1}). On the other hand, introducing both the W191F and W51F mutations significantly improved MnP activity (V_{max}. MnC*c*P(W51F, W191F): 8.0 μmoles min^{-1} mg^{-1}). The increase in activity can be attributed to the W51F mutation since

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MnC*c*P(W51F) (V_{max}: 3.2 μmoles min^{-1} mg^{-1}) showed significantly increased MnP activity relative to MnC*c*P. It is interesting to note that the inclusion of the W51F mutation causes a greater relative increase in activity when the mutant contains the W191F mutation. The $V_{max,MnCcP(W51F)}/V_{max,MnCcP}$ ratio is 4.3 while the $V_{max,MnCcP(W51F,W191F)}/V_{max,MnCcP(W191F)}$ ratio is 14.3.

Table I. Steady-State Kinetic Parameters

Enzyme	V_{max} (μmoles min^{-1} mg^{-1})	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
MnC*c*P	0.75	4.1	0.42	102
MnC*c*P(W191F)	0.56	5.6	0.32	57
MnC*c*P(W51F)	3.20	5.6	1.83	327
MnC*c*P(W51F,W191F)	8.00	7.6	4.55	599
MnP	377.4	0.0732	289.3	3.9 x 10^6

NOTE: Assays for MnC*c*P mutants were performed in 500 mM malonate, pH 5. MnP parameters were obtained in 50 mM malonate, pH 4.5. The activity difference between the MnC*c*P mutants and MnP shown in the table is smaller if compared at the same pH (see text for details.)

SOURCE: Reproduced from Reference 36.

The trend in V_{max} values can be rationalized if one assumes the proposed equilibrium between the Fe(III)Trp+• state and the ferryl state in C*c*P compound II exists. Therefore, compound II is stabilized by both active site tryptophans. W191 stabilizes compound II of C*c*P via the equilibrium and W51 stabilizes compound II by hydrogen bonding to the ferryl oxygen. While delocalization of the radical onto W191 cannot stabilize MnC*c*P(W191F) compound II, it is stabilized by the hydrogen bonding interaction with W51 and no increase in activity is observed. For MnC*c*P(W51F), compound II is stabilized by delocalization of the oxidizing equivalent onto W191 but not by the hydrogen bond from W51 and an increase in activity is observed. For MnC*c*P(W191F,W51F) both possibilities for the stabilization of compound II are absent. Therefore, introducing W51F mutation onto the MnC*c*P(W191F) mutant removes the last stabilizing influence for compound II and the largest activity increase is observed. These significant observations support the hypothesis that the ferryl form of compound II is in equilibrium with form containing ferric Fe and a tryptophan radical.

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The influence of the W51 mutation on the oxidation of ferrocytochrome *c* and aniline derivatives by C*c*P has been reported (*39*). The W51F mutation decreased the stability of C*c*P compound I and increased the k_{cat} for ferrocytochrome *c* oxidation two to four-fold (*25*). These results show that the effects attributed to the W51F mutation here are not unique to the oxidation of Mn(II).

Our results showed that, while the W191F and W51F mutations both play important roles in increasing the lifetime of compound I, the location of the radical in compound I has little influence on MnP activity. The W191F and W51F mutations influence the MnP activity by removing interactions that stabilize compound II. The effect of the W191F mutation is not observed in the MnC*c*P(W191F) mutant because the presence of W51 compensates for the loss of W191 but it is observed for MnC*c*P(W51F, W191F). For MnP, it is known that the reduction of compound II is rate-determining. Likewise, the rate-determining step for the reduction of compound II in C*c*P by ferrocytochrome c is rate-limiting. Our data indicate that the rate-limiting step for Mn(II) oxidation by MnC*c*P is also compound II reduction. In other words, the location of the radical in compound I does not change the rate-limiting step for Mn(II) oxidation by MnC*c*Ps. This conclusion is supported by the direct observation of compound II accumulation under steady-state conditions using UV-visible spectroscopy (Figure 7).

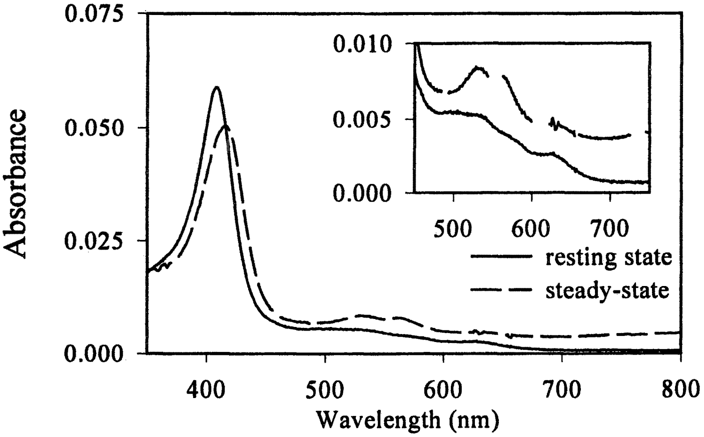

Figure 7. UV-Visible spectra before and after addition of hydrogen peroxide to initiate stead-state catalysis.

It is interesting to note that the number of tryptophans (seven) and tyrosines (fourteen) in C*c*P is unusually high for a protein of its size (34KD) while MnP

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lacks tyrosine residues and contains only one tryptophan. It could be assumed that tryptophans and tyrosines are therefore detrimental to the proper functioning of MnP while W191 is critical to the proper functioning of C*c*P. Since the Mn(III) product must diffuse from the active site and react with lignin, side reactions where the protein reduces the Mn(III) to Mn(II) must be avoided. Tyrosines and tryptophans could potentially serve as reductants. This notion is supported by the data for Mn(II) oxidation catalyzed by MnC*c*P(W51F, W191F). Following the initial burst of product generation that results from addition of hydrogen peroxide, the concentration of Mn(III)-malonate (determined by the absorption at 270 nm) decreases. This presumably occurs due to reduction of the reactive Mn(III) complex. The tryptophans and tyrosines located in C*c*P could reduce the Mn(III) chelator complex. However, the reaction of Mn(III) with the excess hydrogen peroxide present in the steady-state reaction cannot be ruled out.

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In summary, we have successfully engineered a Mn(II)-binding site into cytochrome *c* peroxidase (C*c*P) (*34*). The binding of Mn(II) to MnC*c*P was demonstrated using UV-visible absorption (UV-vis) and paramagneticallly-shifted proton NMR spectroscopies (*35*). In combination, these results strongly suggest that Mn(II) binds specifically to the engineered binding site in MnC*c*P. The engineered Mn(II)-binding site mimics the site in MnP including the "wiring" of the Mn(II) site to the oxidizing equivalents via coordination of a heme propionate. Most importantly, the engineered Mn(II)-binding site facilitates Mn(II) oxidation by C*c*P (V_{max} = 0.75 μmoles $min^{-1}mg^{-1}$). The manganese peroxidase activity of our model system was further improved by incorporating the W191F and W51F mutations (*36*). These mutations improved the structural similarity in heme pocket between our model and MnP and increased the activity (V_{max} = 8.0 μmoles min^{-1} mg^{-1}). From this study, we showed that, even though both mutations increase the lifetime of compound I, the W51F mutation contributes much more to the increase of MnP activity because it increases the reactivity of compound II with Mn(II).

The work described here demonstrated that designing a MnP from a structurally similar but functionally different protein C*c*P can result in structural and mechanistic insight into the enzyme function that complements results obtained from the study of the native enzyme and synthetic model systems. This protein model may provide a cheaper alternative catalyst for industrial applications. When measured at similar conditions as native MnP, the specific activity of MnC*c*P(W191F, W51F) is only one order of magnitude less than that of native MnP (at pH 4.5, V_{max} = 20 μmoles min^{-1} mg^{-1} for MnC*c*P(W191F, W51F) and V_{max} = 377 μmoles min^{-1} mg^{-1} for MnP). This is remarkable considering that MnC*c*P(W191F, W51F) contains only five mutations out of 294 amino acid residues, >80% of which are different from MnP. Further improvement of this protein model system includes refinement of the Mn(II)-binding site to improve K_M and k_{cat} as well as elimination of other tryptophans and tyrosines. Progress has been made in this direction and will be reported in the future.

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